EXHIBIT 1

EEOC Form 5 (5/01)

CHARGE OF DISCRIMINATION

This form is affected by the Privacy Act of 1974. See enclosed Privacy Act Statement and other information before completing this form.

Charge Presented To: [ ] FEPA [X] EEOC

Agency(ies) Charge No(s): 532-2007-00194

Ohio Civil Rights Commission and EEOC

State or local Agency, if any

Name (indicate Mr., Ms., Mrs.)	Home Phone (Incl. Area Code)	Date of Birth
Ms. Sheryl Szeinbech	(614) 529-1996	09-26-1956

Street Address	City, State and ZIP Code
P.O. Box 21323	Columbus, OH 43221

Named is the Employer, Labor Organization, Employment Agency, Apprenticeship Committee, or State or Local Government Agency That I Believe Discriminated Against Me or Others. (*If more than two, list under PARTICULARS below.*)

Name	No. Employees, Members	Phone No. (Include Area Code)
OHIO STATE UNIVERSITY, College of Pharmacy	500 or More	(614) 292-2800

Street Address	City, State and ZIP Code
500 West 12th Avenue	Columbus, OH 43210

Name	No. Employees, Members	Phone No. (Include Area Code)

Street Address	City, State and ZIP Code

DISCRIMINATION BASED ON (*Check appropriate box(es).*)

[ ] RACE [ ] COLOR [X] SEX [ ] RELIGION [ ] NATIONAL ORIGIN

[X] RETALIATION [ ] AGE [ ] DISABILITY [ ] OTHER (*Specify below.*)

DATE(S) DISCRIMINATION TOOK PLACE

Earliest: 09-01-2006 Latest: 09-30-2006

[X] CONTINUING ACTION

THE PARTICULARS ARE (*If additional paper is needed, attach extra sheet(s)*):

I was hired on January 19, 1999 by the above named Respondent. My current position is Tenure Professor. In September 2006, I was subjected to retaliation. discrimination

In August 2006 and prior, I complained to Bob Bruggermier, Dean, of his discriminatory treatment of a male colleague. In September 2006, I received a below average performance rating and a low salary increase, although previous wage increases were higher and performance ratings were good.

I believe I was retaliated against because of my sex, female, and opposing of discriminatory practices in violation of Title VII of the Civil Rights Act of 1964, as amended.

I want this charge filed with both the EEOC and the State or local Agency, if any. I will advise the agencies if I change my address or phone number and I will cooperate fully with them in the processing of my charge in accordance with their procedures.

I declare under penalty of perjury that the above is true and correct.

11/22/06
Date

Charging Party Signature

NOTARY – *When necessary for State and Local Agency Requirements*

I swear or affirm that I have read the above charge and that it is true to the best of my knowledge, information and belief.
SIGNATURE OF COMPLAINANT

SUBSCRIBED AND SWORN TO BEFORE ME THIS DATE
(*month, day, year*)

EXHIBIT 2

EEOC Form 161-B (3/98)

U.S. EQUAL EMPLOYMENT OPPORTUNITY COMMISSION

NOTICE OF RIGHT TO SUE *(ISSUED ON REQUEST)*

To: **Sheryl L. Szeinbach, PhD**
P.O. Box 21323
Columbus, OH 43221

From: **Cleveland Field Office**
AJCFB - Suite 3001
1240 E. 9th St
Cleveland, OH 44199

☐ *On behalf of person(s) aggrieved whose identity is CONFIDENTIAL (29 CFR §1601.7(a))*

EEOC Charge No.	EEOC Representative	Telephone No.
532-2007-02009	**Legal Unit, Duty Officer**	**(216) 522-7445**

(See also the additional information enclosed with this form.)

NOTICE TO THE PERSON AGGRIEVED:

Title VII of the Civil Rights Act of 1964 and/or the Americans with Disabilities Act (ADA): This is your Notice of Right to Sue, issued under Title VII and/or the ADA based on the above-numbered charge. It has been issued at your request. Your lawsuit under Title VII or the ADA **must be filed in a federal or state court WITHIN 90 DAYS of your receipt of this notice**; or your right to sue based on this charge will be lost. (The time limit for filing suit based on a state claim may be different.)

☒ More than 180 days have passed since the filing of this charge.

☐ Less than 180 days have passed since the filing of this charge, but I have determined that it is unlikely that the EEOC will be able to complete its administrative processing within 180 days from the filing of this charge.

☒ The EEOC is terminating its processing of this charge.

☐ The EEOC will continue to process this charge.

Age Discrimination in Employment Act (ADEA): You may sue under the ADEA at any time from 60 days after the charge was filed until 90 days after you receive notice that we have completed action on the charge. In this regard, **the paragraph marked below applies to your case:**

☐ The EEOC is closing your case. Therefore, your lawsuit under the ADEA **must be filed in federal or state court WITHIN 90 DAYS of your receipt of this Notice.** Otherwise, your right to sue based on the above-numbered charge will be lost.

☐ The EEOC is continuing its handling of your ADEA case. However, if 60 days have passed since the filing of the charge, you may file suit in federal or state court under the ADEA at this time.

Equal Pay Act (EPA): You already have the right to sue under the EPA (filing an EEOC charge is not required.) EPA suits must be brought in federal or state court within 2 years (3 years for willful violations) of the alleged EPA underpayment. This means that **backpay due for any violations that occurred more than 2 years (3 years) before you file suit may not be collectible.**

If you file suit, based on this charge, please send a copy of your court complaint to this office.

On behalf of the Commission

Daniel Cabot/EP

Daniel Cabot,
Field Office Director

JUL 24 2008
(Date Mailed)

Enclosures(s)

cc: **Olga Esquivel-Gonzalez, JD**
Employment Law & Compliance Manager
OHIO STATE UNIVERSITY
Office of Human Resources
1590 North High Street, #300
Columbus, OH 43201-2190

Eric J. Rosenberg, Esq.
Rosenberg Law Office, Co. LPA
395 North Pearl Street
Granville, Ohio 43023

Enclosure with EEOC
Form 161-B (3/96)

INFORMATION RELATED TO FILING SUIT UNDER THE LAWS ENFORCED BY THE EEOC

*(This information relates to filing suit in Federal or State court under Federal law.
If you also plan to sue claiming violations of State law, please be aware that time limits and other provisions of State law may be shorter or more limited than those described below.)*

PRIVATE SUIT RIGHTS -- Title VII of the Civil Rights Act, the Americans with Disabilities Act (ADA), or the Age Discrimination in Employment Act (ADEA):

In order to pursue this matter further, you must file a lawsuit against the respondent(s) named in the charge **within 90 days of the date you *receive* this Notice**. Therefore, you should **keep a record of this date**. Once this 90-day period is over, your right to sue based on the charge referred to in this Notice will be lost. If you intend to consult an attorney, you should do so promptly. Give your attorney a copy of this Notice, and its envelope, and tell him or her the date you received it. Furthermore, in order to avoid any question that you did not act in a timely manner, it is prudent that your suit be filed **within 90 days of the date this Notice was *mailed* to you** (as indicated where the Notice is signed) or the date of the postmark, if later.

Your lawsuit may be filed in U.S. District Court or a State court of competent jurisdiction. (Usually, the appropriate State court is the general civil trial court.) Whether you file in Federal or State court is a matter for you to decide after talking to your attorney. Filing this Notice is not enough. You must file a "complaint" that contains a short statement of the facts of your case which shows that you are entitled to relief. Your suit may include any matter alleged in the charge or, to the extent permitted by court decisions, matters like or related to the matters alleged in the charge. Generally, suits are brought in the State where the alleged unlawful practice occurred, but in some cases can be brought where relevant employment records are kept, where the employment would have been, or where the respondent has its main office. If you have simple questions, you usually can get answers from the office of the clerk of the court where you are bringing suit, but do not expect that office to write your complaint or make legal strategy decisions for you.

PRIVATE SUIT RIGHTS -- Equal Pay Act (EPA):

EPA suits must be filed in court within 2 years (3 years for willful violations) of the alleged EPA underpayment: back pay due for violations that occurred **more than 2 years (3 years) before you file suit** may not be collectible. For example, if you were underpaid under the EPA for work performed from 7/1/00 to 12/1/00, you should file suit before 7/1/02 – *not* 12/1/02 -- in order to recover unpaid wages due for July 2000. This time limit for filing an EPA suit is separate from the 90-day filing period under Title VII, the ADA or the ADEA referred to above. Therefore, if you also plan to sue under Title VII, the ADA or the ADEA, in addition to suing on the EPA claim, suit must be filed within 90 days of this Notice and within the 2- or 3-year EPA back pay recovery period.

ATTORNEY REPRESENTATION -- Title VII and the ADA:

If you cannot afford or have been unable to obtain a lawyer to represent you, the U.S. District Court having jurisdiction in your case may, in limited circumstances, assist you in obtaining a lawyer. Requests for such assistance must be made to the U.S. District Court in the form and manner it requires (you should be prepared to explain in detail your efforts to retain an attorney). Requests should be made well before the end of the 90-day period mentioned above, because such requests do not relieve you of the requirement to bring suit within 90 days.

ATTORNEY REFERRAL AND EEOC ASSISTANCE -- All Statutes:

You may contact the EEOC representative shown on your Notice if you need help in finding a lawyer or if you have any questions about your legal rights, including advice on which U.S. District Court can hear your case. If you need to inspect or obtain a copy of information in EEOC's file on the charge, please request it promptly in writing and provide your charge number (as shown on your Notice). While EEOC destroys charge files after a certain time, all charge files are kept for at least 6 months after our last action on the case. Therefore, if you file suit and want to review the charge file, **please make your review request within 6 months of this Notice**. (Before filing suit, any request should be made within the next 90 days.)

IF YOU FILE SUIT, PLEASE SEND A COPY OF YOUR COURT COMPLAINT TO THIS OFFICE.

EXHIBIT 3

Brueggemeier.1@osu.edu, 02:07 PM 2/0/2005 IMPORTANT

To: Brueggemeier.1@osu.edu
From: Sheryl Szeinbach <szeinbach.1@osu.edu>
Subject: IMPORTANT
Cc:
Bcc:
Attached:

Dear Bob: I attended the P & T meeting yesterday. I have questions regarding the fairness of the evaluation that was performed for Enrique Seoane-Vazquez. I felt the presentation of the evaluation was intentionally very biased against Enrique – there was a lot of discussion as well. I was wondering if Enrique should be evaluated at all last year given his extensive illness, where his recovery took several months. Also, I wanted to provide a message *a priori* so there is an awareness of the situation ---

I would not send this message unless I felt very strongly that something is not right—

Thanks so much, Sheryl

Sheryl L. Szeinbach, Ph.D.
Professor, Division of Pharmacy
Practice and Administration
The Ohio State University
College of Pharmacy
500 W. 12th Avenue
Columbus, OH 43210-1291
614-688-4249 (o)
614-292-1335 (f)
zbach@dendrite.pharmacy.ohio-state.edu
Szeinbach.1@osu.edu

EXHIBIT 4

X-Original-To: szeinbach@pharmacy.ohio-state.edu
Delivered-To: szeinbach@pharmacy.ohio-state.edu
Date: Wed, 08 Nov 2006 16:09:36 -0500
From: Jim Dalton <dalton.1@osu.edu>
Subject: Re: Record of Review for Dr. Enrique Seoane-Vazquez
To: Sheryl Szeinbach <szeinbach.1@osu.edu>
Cc: brueggemeier.1@osu.edu
X-Mailer: QUALCOMM Windows Eudora Version 7.0.1.0
X-MIMEDefang-Relay-89167b66339720c294cd81d33948afd6488b114f: 128.146.81.96
X-Bayes-Prob: 0.9999 (Score 15)
X-CanIt-Tag-Reason: score = 15.9; probability = 0.9999; hold_reason = SpamScore
X-Spam-Score: 15.90 (**********) [Tag at 10.00] HTML_10_20,HTML_MESSAGE
X-CanItPRO-Stream: 20_low
X-CanIt-Stats-ID: 130835011 - 19a42f454621
X-Scanned-By: CanIt (www . roaringpenguin . com) on 128.146.216.134
X-Authentication-warning: defang20.it.ohio-state.edu: defang set sender to <dalton.1@osu.edu> using -f

Dear Sheryl:
As Dean Brueggemeier announced in the College P&T meeting today, we will not be considering the 4th year review case for Dr. Seoane-Vasquez at this time (i.e., November 2006) due to a procedural issue. Instead, the process has been re-started, and all previous information, including the dossier, chair's letter, etc will be discarded. A separate meeting for this case with revised materials from the candidate, division, and chair will be held early next year. We collected all copies of Dr. Seoane-Vasquez's dossier from eligible faculty, with the exception of yours. Please return your copy of the dossier to me by 3 PM tomorrow (i.e., prior to the scheduled P&T committee). Thanks.
Jim

At 04:48 PM 11/3/2006, you wrote:

> Dear Jim: I wanted to provide the following information to you --
>
> Thanks, Sheryl
>
> James Dalton, Ph.D.
> Chair, Appointments, Promotion and Tenure committee
> College of Pharmacy
> 242 Parks Hall
> 500 West 12th Avenue
> Columbus, OH 43210
>
> Re: Inaccuracies in the Record of Review for Promotion in Academic Rank-Tenure-Reappointment for Dr. Enrique Seoane-Vazquez.
>
> Dear Dr. Dalton:

After examining the materials provided to me by Gail Vornholt on October 24, 2006 for the fourth year review of Dr. Enrique Seoane-Vazquez, I note inaccuracies in the fourth year review letter and faculty reviews provided by Dr. Milap Nahata.

Fourth Year Review Letter

1. Page 2. No documentation was ever made available to senior faculty in support of the statement that faculty in the management sequence had higher SEI scores than the 4.1 score obtained by Dr. Seoane during 2005-2006.

2. Page 3. The abstract for the ISPOR meeting was accepted for presentation. Dr. Seoane listed this abstract in his dossier as accepted - not presented. However, at the last minute the graduate student made changes to the poster, these changes were unacceptable to both Dr. Seoane and Dr. Szeinbach. The graduate student - now a faculty member at Finley - was asked to remove our names from the poster if he planned to present the material with unauthorized changes.

3. Page 4. Dr. Nahata states for the fourth year review, "eight of the nine senior faculty members of the Division ranked Dr. Seoane's performance in teaching and research/scholarship below a satisfactory level." This ranking was actually a Division faculty vote conducted by a paper ballot.

Faculty Review - 2004

It is stated that "all ten tenured faculty (associate and full professors) discussed his performance and concurred that Enrique is not making progress as a tenure-track faculty member at the Ohio State."

This is not true I did not concur with this statement.

Sheryl L. Szeinbach, Ph.D.
Professor, Division of Pharmacy Practice & Administration

Sheryl L. Szeinbach, Ph.D.
Professor, Division of Pharmacy
Practice and Administration
The Ohio State University
College of Pharmacy
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614-292-1335 (f)
zbach@dendrite.pharmacy.ohio-state.edu
Szeinbach.1@osu.edu

EXHIBIT 5

> >
> >
> >Dear Dr. Holmes:
> >
> >This is a serious issue and hence I am ccing you on this as the
> chair of
> >the group that publishes this journal.
> >
> >RB
> >
> >---------- Original Message ----------
> --------
> >Subject: [Fwd: 2 research reports with same results and different
> >co-authors]
> >From: balkrishnan@pharmacy.ohio-state.edu
> >Date: Sat, April 28, 2007 12:53 pm
> >To: marklevy@animalswild.com
> >----------
> --------
> >
> >Dear Dr. Levy-
> >
> >I am writing to you in confidence to point this out.
> >I have come across 2 research reports, one published in
> >2005 and the other in 2007 (in your journal) in different
> journals with
> >exactly identical results just analyzing the data slightly
> differently. Is
> >this something which is OK? Both papers and abstracts are
> attached for
> >your
> >reference. Also the 2007 paper does not reference the 2005 paper.
> I am
> >surprised that this was not picked up in peer review.
> >
> >Thanks
> >
> >
> >RB
> >
> >----------
> --------

EXHIBIT 6

meet with other senior editors in the respiratory world and I believe they work strongly as a group to investigate this type of incident.

Many thanks for notifying me - and I am glad that I am able to respond positively.

Best wishes

Steve
Dr Steve Holmes
MMedSci, MBChB, FRCGP
Chair General Practice Airways Group
Chair Severn Faculty RCGP
RCGP Council Member
GP Shepton Mallet

---------- Original Message ----------
Subject: 2 research reports with same results and different co-authors
From: balkrishnan@pharmacy.ohio-state.edu
Date: Sun, April 29, 2007 2:46 pm
To: mcgrath.66@osu.edu
snyder.7@osu.edu
Cc: nahata.1@osu.edu
brueggemeier.1@osu.edu

Dear Dr. McGrath and Dr. Snyder:

Please find attached along with the OSU whistleblower form which is required by OSU human resources.

RB

---------- Original Message ----------
Subject: 2 research reports with same results and different co-authors
From: balkrishnan@pharmacy.ohio-state.edu
Date: Sat, April 28, 2007 11:20 am
To: nahata.1@osu.edu

Milap-

Just wanted to get your opinion on this being the editor in chief of a major journal. I have come across 2 research reports, one published in 2005 and the other in 2007 in different journals with exactly identical results just analyzing the data slightly differently. Is this something which is OK? How would something like this be construed at the Annals of Pharmacotherapy? Both papers and abstracts are attached for your reference. Also the 2007 paper does not reference the 2005 paper.

Thanks

RB

2007

Primary Care Respiratory Journal

Volume 16 Issue 2 April 2007
The impact of allergic rhinitis on work productivity Â Original Research

Pages 98-105

Sheryl L Szeinbach,*, Enrique C Seoane-Vazquez, Andrew Beyer, P Brock Williams

a College of Pharmacy, Ohio State University, Columbus, Ohio, USA
b University of Missouri Medical School, Kansas City, Missouri, USA

EXHIBIT 7

Date: Tue, 01 May 2007 13:07:35 -0400
From: Rajesh Balkrishnan <balkrishnan@pharmacy.ohio-state.edu>
Subject: Re: Fwd: RE: [Fwd: 2 research reports with same results and different co-authors]
To: Craig Pedersen <pedersen.18@osu.edu>, nahata.1@osu.edu, brueggemeier.1@osu.edu, hayton.1@osu.edu
X-Mailer: QUALCOMM Windows Eudora Version 7.0.1.0
X-MIMEDefang-Relay-89167b66339720c294cd81d33948afd6488b114f: 128.146.80.130
X-Spam-Score: 0.90 () [Tag at 4.50] HTML_10_20,HTML_MESSAGE
X-CanItPRO-Stream: 11_tagonly_no_subject
X-Canit-Stats-ID: Bayes signature not available
X-Scanned-By: CanIt (www . roaringpenguin . com) on 128.146.216.21
X-Authentication-warning: defang12.it.ohio-state.edu: defang set sender to <balkrishnan@pharmacy.ohio-state.edu> using -f
Original-recipient: rfc822;pedersen.18@osu.edu

I will defer to the rest of the group for the final decision, but I will respectfully disagree with Craig about this for issues I can discuss with each of you who may need an explanation.

RB

At 12:12 PM 5/1/2007, Craig Pedersen wrote:

> Raj, Milap, Bob, and Bill:
>
> Interesting. In know that a student is involved in this [redacted] but it is my recommendation to keep him out of this. He may not be aware, or may not know, or may have been influenced, or some other explanation. This is an issue with faculty in my opinion. Lets see what the editors of the 2007 publication have to say before proceeding further.
>
> Craig
>
> At 11:25 AM 5/1/2007, Rajesh Balkrishnan wrote:
>
> > X-Original-To: balkrishnan@pharmacy.ohio-state.edu
> > Delivered-To: balkrishnan@pharmacy.ohio-state.edu
> > Subject: RE: [Fwd: 2 research reports with same results and different co-authors]
> > Date: Tue, 1 May 2007 15:49:23 +0100
> > Thread-Topic: [Fwd: 2 research reports with same results and different co-authors]
> > thread-index: AceJ1KNsRpOewCSRScihMXLMOTRwWQCKYIjQ
> > From: "Steve Holmes" <Steve.Holmes@parkmedicalpractice.nhs.uk>
> > To: <balkrishnan@pharmacy.ohio-state.edu>
> > Cc: <marklevy@animalswild.com>
> >
> > Dear RB,
> > Thanks for copying me into this. I am aware as Dr Mark Levy and Dr Paul Stephenson have copied me into other correspondance. I agree this is very serious and is being dealt with as such. I am aware that discussions have gone on between the original publishers and background work on the consent we were given indicating that the work had not been published elsewhere. You can be assured that this is something that will be progressed by

the PCRJ robustly through the appropriate channels. Our senior editorial team meet with other senior editors in the respiratory world and I believe they work strongly as a group to investigate this type of incident.

Many thanks for notifying me - and I am glad that I am able to respond positively.

Best wishes

Steve
Dr Steve Holmes
MMedSci, MBChB, FRCGP
Chair General Practice Airways Group
Chair Severn Faculty RCGP
RCGP Council Member
GP Shepton Mallet

-----Original Message-----
From: balkrishnan@pharmacy.ohio-state.edu
[mailto:balkrishnan@pharmacy.ohio-state.edu]
Sent: 28 April 2007 21:16
To: Steve Holmes
Subject: [Fwd: 2 research reports with same results and different co-authors]
Importance: High

Dear Dr. Holmes:

This is a serious issue and hence I am ccing you on this as the chair of the group that publishes this journal.

RB

---------- Original Message ----------
Subject: [Fwd: 2 research reports with same results and different co-authors]
From: balkrishnan@pharmacy.ohio-state.edu
Date: Sat, April 28, 2007 12:53 pm
To: marklevy@animalswild.com

Dear Dr. Levy-

I am writing to you in confidence to point this out.
I have come across 2 research reports, one published in 2005 and the other in 2007 (in your journal) in different journals with exactly identical results just analyzing the data slightly differently. Is this something which is OK? Both papers and abstracts are attached for

your
reference. Also the 2007 paper does not reference the 2005 paper. I am surprised that this was not picked up in peer review.

Thanks

RB

2007

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The impact of allergic rhinitis on work productivity * Original Research

Pages 98-105

Sheryl L Szeinbach,*, Enrique C Seoane-Vazquez, Andrew Beyer, P Brock Williams

a College of Pharmacy, Ohio State University, Columbus, Ohio, USA
b University of Missouri Medical School, Kansas City, Missouri, USA

Abstract
AIM: Patients with allergic rhinitis experience a multitude of symptoms that usually compromise some aspect of lifestyle. However, few data are available that specifically address the impact of allergic rhinitis on work productivity. METHODS: A questionnaire was developed and mailed to 2,065 patients enrolled in a 500,000-member managed care organisation. Patients were identified by diagnostic codes for allergic rhinitis as determined by a retrospective examination of medical and prescription claims records from January 1 2000 to December 31 2000. Patients were divided into three different care groups according to whether they were managed by family physicians, by allergists, or were self-managed. RESULTS: Chi-square and analysis of variance tests revealed significant differences among the three care groups ($p<0.05$) for years with allergies, symptoms, family history, testing, immunotherapy, test value, and prescribed antihistamine use. Multiple linear regression analysis revealed that sleep, health, certain allergy symptoms and prescribed antihistamines were significantly related to work productivity. CONCLUSIONS: The results of this study revealed that the ability of individuals with allergic rhinitis to engage in productive work is influenced by sleep, health-related quality of life (HRQoL), specific symptoms, and prescribed antihistamine use.

EXHIBIT 8

To: Dr. Jennifer Mosely
614-688-0366

From. Dr. Sheryl Szeinbach

-----Original Message-----
From: balkrishnan@pharmacy.ohio-state.edu [mailto:balkrishnan@pharmacy.ohio-state.edu]
Sent: Tuesday, May 01, 2007 1:07 PM
To: fshaya@rx.umaryland.edu; pneumann@tufts-nemc.org; Diana Brixner; bmartin@uams.edu; fpapath@ucsd.edu
Subject: Very Important!
Importance: High

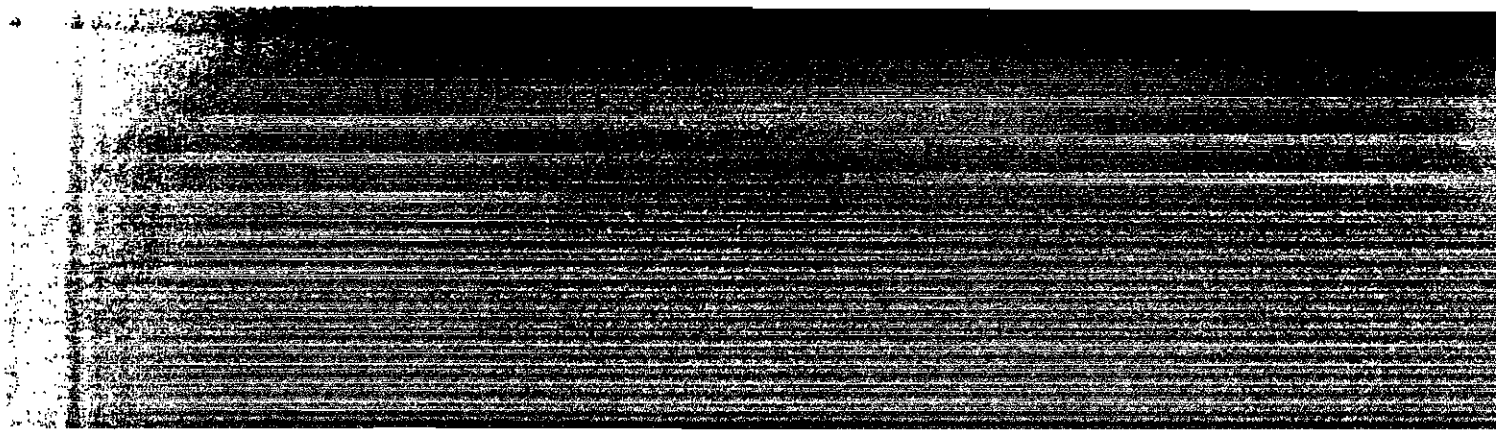
This research is being presented as new research at the ISPOR 2007 meeting. Also this identical research has been previously presented at an ISPOR meeting as well (2005).
I just wanted you all to be aware of this before it is presented again.

RB

From: "Steve Holmes" <Steve.Holmes@parkmedicalpractice.nhs.uk>
To: <balkrishnan@pharmacy.ohio-state.edu>
Cc: <markdevy@animalswild.com>

Dear RB,
Thanks for copying me into this. I am aware as Dr Mark Levy and Dr Paul Stephenson have copied me into other correspondance. I agree this is very serious and is being dealt with as such. I am aware that discussions have gone on between the original publishers and background work on the consent we were given indicating that the work had not been published elsewhere. You can be assured that this is something that will be progressed by the PCRI robustly through the appropriate channels. Our senior editorial team

[redacted] 6/13/2007



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Best wishes

Steve
Dr Steve Holmes
MMedSci, MBChB, FRCGP
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Cc: nahata.1@osu.edu
brueggemeier.1@osu.edu

Dear Dr. McGrath and Dr. Snyder:

Please find attached along with the OSU whistleblower form which is required by OSU human resources.

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Subject: 2 research reports with same results and different co-authors
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Thanks

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a College of Pharmacy, Ohio State University, Columbus, Ohio, USA
b University of Missouri Medical School, Kansas City, Missouri, USA

EXHIBIT 9

X-Original-To: szeinbach@pharmacy.ohio-state.edu
Delivered-To: szeinbach@pharmacy.ohio-state.edu
Reply-To: <marklevy@animalswild.com>
From: "Mark Levy" <mark-levy@btconnect.com>
To: "'Szeinbach'" <szeinbach@pharmacy.ohio-state.edu>
Subject: RE: Editorial
Date: Wed, 15 Aug 2007 07:20:27 +0100
X-Mailer: Microsoft Office Outlook 12.0
Thread-Index: AcfevEGm3pTCHHCKTW2tWCImZY3q6wARwPPw

Dear Sheryl

I can only apologise for this oversight, I did mean to do so..
It is unfortunate that the letter below has been sent out. This letter misinterprets and overstates the contents of our carefully written editorial, which concluded that although there was an oversight, this was not intentional. We have also published your correction at www.thepcrj.org, which is self explanatory.

Please copy this note from me to those in your department if you wish.

DR Mark L Levy FRCGP
Editor-in-Chief PCRJ.

Dr Mark L Levy FRCGP
Senior Clinical Research Fellow: Allergy & Respiratory Research Group
Division of Community Health Sciences, University of Edinburgh
www.consultmarklevy.com

Wildlife Photography (images, prints and posters)
- **www.animalswild.com**
- http://gallery.artistrising.com/HomePage/ArtistHomePage.aspx?AIID=28227
- www.alamy.com/stock-photography-contrib-browse.asp?cid=%7b11AF87AC-A4A7-4F77-A072-82D0A0ABD7B8%7d&name=Mark+Levy

From: Szeinbach [mailto:szeinbach@pharmacy.ohio-state.edu]
Sent: 14 August 2007 22:59
To: Mark Levy
Subject: Editorial

Dear Dr. Levy:

EXHIBIT 10

I thought I was going to receive a copy of the editorial from you when it was published?

That's okay -- Raj Balkrishnan was kind enough to send a copy of the editorial to me and everyone in the College of Pharmacy --

X-Original-To: zbach@pharmacy.ohio-state.edu
Delivered-To: szeinbach@tetraknot.pharmacy.ohio-state.edu
Delivered-To: facppad@tetraknot.pharmacy.ohio-state.edu
X-Mailer: QUALCOMM Windows Eudora Version 7.0.1.0
Date: Mon, 13 Aug 2007 12:35:16 -0400
To: faculty@pharmacy.ohio-state.edu
From: Rajesh Balkrishnan <balkrishnan@pharmacy.ohio-state.edu>
Subject: For your information!
List-Unsubscribe: <mailto:leave-facppad-211U@rx8.pharmacy.ohio-state.edu>

Dear faculty members-

I am extremely saddened to report that a major clinical journal has published this. This is a matter of great shame and disrepute to the Ohio State University College of Pharmacy.

Thank you very much for your attention to this.

Rajesh Balkrishnan, PhD
Merrell Dow Professor
Ohio State University College of Pharmacy and School of Public Health
500 W. 12th Avenue, Columbus, OH 43210
Phone: 614-292-6415
Fax: 614-292-1335
Email: balkrishnan.1@osu.edu
http://myprofile.cos.com/rbalkrishnan
View my lab at: http://www.pharmacy.ohio-state.edu/programs/ppad/labs/CeuticalOutcomesLab.cfm
ams

Sheryl

Sheryl L. Szeinbach, Ph.D., R.Ph.
Professor, Division of Pharmacy
Practice and Administration
Ohio State University
College of Pharmacy
500 W. 12th Ave.
Columbus, OH 43210-1291
614-688-4249 (O)
Szeinbach.1@osu.edu

EXHIBIT 11

** Replied on Wed 8/22/2007 10:10 AM **

From: Szeinbach (szeinbach@pharmacy.ohio-state.edu)
Sent: Tue 8/21/2007 08:27 PM
Rcvd: Tue 8/21/2007 08:12 PM
To: David T. Ball (DBALL)
Subject: Fwd: Re: Editor's response and my response to August 13th email

---

X-Original-To: szeinbach@pharmacy.ohio-state.edu
Delivered-To: szeinbach@pharmacy.ohio-state.edu
Date: Tue, 21 Aug 2007 18:14:34 -0400 (EDT)
Subject: Re: Editor's response and my response to August 13th email
From: balkrishnan@pharmacy.ohio-state.edu
To: "Szeinbach" <szeinbach@pharmacy.ohio-state.edu>
Cc: faculty@pharmacy.ohio-state.edu, szeinbach.1@osu.edu
User-Agent: SquirrelMail/1.4.8-6.el3

I apologize for the previous email which was sent incompletely-RB

FYR, Sheryl, and the Pharmacy faculty-

I think it is rather unfortunate that Dr. Szeinbach has taken this whole issue so personally. According to her email "I believe that Dr. Balkrishnan has chosen to bring this matter to your attention in an attempt to undermine the reputations of Dr. Enrique Seoane-Vazquez and me, and that this is a further example of Dr. Balkrishnan's discrimination and retaliation against us."

This is rather unfortunate because my email does not refer to Dr. Szeinbach or Dr. Seoane-Vazquez at all. Rather my concern is that in my opinion, the reputation of the college has been compromised when an editorial is published in a major journal which states the following: "However there is no doubt in our mind....Furthermore in not referencing the AAAI paper……later PCRJ context"

Also my initial email to the concerned authorities was in the interest of scientific integrity and not to single out any particular faculty for discrimination as the following emails will show:

I also enclose a copy of my exact letter to Dr. Levy and concerned authorities in the OSU whistleblower report form:

I have sent the following email to the attention of chair of the department of pharmacy administration, and cced to the associate dean for research in the college of pharmacy, director of graduate studies (since the research was presented at new research at the graduate seminar conducted and coordinated by the director of graduate studies and attended by all faculty members), the editors of the 2 journals, office of academic affairs and the Vice president of research. I am not alleging any wrongful conduct, but as a member of a responsible research community would like an answer to this question:
Just wanted to get your opinion on this being the editor in chief of a major journal. I have come across 2 research reports, one published in 2005 and the other in 2007 in different journals with exactly identical results just analyzing the data slightly differently. Is this something which is OK? How would something like this be construed at the Annals of Pharmacotherapy? Both papers and abstracts are attached for your reference. Also the 2007 paper does not reference the 2005 paper.

Thanks

RB

Also this is another response I got from Dr. Gailen Marshall after I sent him an email stating that I had conferred with 3 other scientists who were not at OSU or who did not know Dr. Szeinbach, who felt that this was a serious matter of near duplication that had to be reported. I followed due process before sending this email. Here is Dr. Marshalls email to me after he received my query with appropriate feedback from 3 Non-OSU faculty:

>Dr. Balkrishnan - thank you again for your candor and integrity. I
>am so very saddened when something like this happens because of its
>potential impact on the scientific process. Yet I realize that
>people are people and none of us are beyond temptation. Having said
>that, this is a significant research ethical issue which must be and
>will be dealt with in a forceful way. The message must clearly be
>sent that such behavior by any of us is totally unacceptable--but
>most importantly wher it involves trainees. Hopefully, we can send a
>forceful message that will have value to both Dr. Szeinbach, her
>trainees and the research community as a whole.
>
>Please be assured that your identity will be kept confidential from
>my standpoint.
>
>Kind regards,

>
>gailen
>
>Gailen D. Marshall, MD PhD
>Editor-in-chief
>Annals of Allergy, Asthma and Immunology
>
>Professor of Medicine and Pediatrics
>Vice Chair for Faculty Development
>Director, Division of Clinical Immunology and Allergy
>The University of Mississippi Medical Center
>2500 North State Street
>Jackson, MS 39216-4505

Sheryl L. Szeinbach, Ph.D., R.Ph.
Professor, Division of Pharmacy
Practice and Administration
Ohio State University
College of Pharmacy
500 W. 12th Ave.
Columbus, OH 43210-1291
614-688-4249 (O)
Szeinbach.1@osu.edu

EXHIBIT 12

THE ENDOCRINE SOCIETY

The Journal of
Clinical Endocrinology & Metabolism

Editorial Office
The Endocrine Society
8401 Connecticut Avenue, Suite 900
Chevy Chase, MD 20815-5817 USA
Phone: (301) 951-2615
Fax: (301) 951-2617
Email: sherman@endo-society.org
Web: http://jcem.endojournals.org/

March 18, 2008

Robert Brueggemeier, Ph.D.
The Ohio State University
College of Pharmacy
500 West 12th Avenue
1960 Kenny Rd
Columbus, OH 43210-1291

Dear Dr. Brueggemeier:

We have completed an initial review of 1) the specific allegations made regarding your dual publications of data and 2) your explanation to us. We conclude that there remain two significant problems not satisfactorily explained by your response.

First, you submitted as original work for your 2003 JCEM article data that had been previously published in a minimally different format in the Journal of Steroid Biochemistry & Molecular Biology [December 2001] 79:75-84.

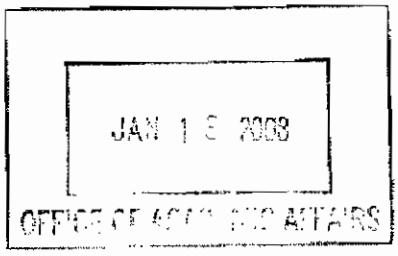
Second, several figures first published in JCEM in 2005, subsequently appeared with minimal revision in the Journal of Steroid Biochemistry & Molecular Biology [May 2005] 95:129-136 and Anti-Cancer Agents in Medicinal Chemistry [May 2006] 6:221-232. To our knowledge, you failed to seek permission from our journal for use of this copyrighted material. Although the JCEM publication is generally cited in the reference list, it is not denoted in the legend to these figures.

Consequently, we are taking the following actions. First, we request an apology from you to the Journal and the Endocrine Society for your actions. Second, we ask that you provide us with the name of the responsible academic officer at Ohio State, so they can determine if these instances were among those previously investigated by your institution. We will leave further pursuit of these matters to them.

Yours truly,

Paul W. Ladenson, M.D.
Editor-in-Chief, Journal of Clinical Endocrinology and Metabolism

EXHIBIT 13



Enrique C. Seoane-Vazquez
Assistant Professor
College of Pharmacy and
School of Public Health
The Ohio State University
500 West 12th Avenue
Room 129 B
Columbus, Ohio 43210

January 18, 2008

Joseph A. Alutto
Executive Vice President and Provost
Office of Academic Affairs
203 Bricker Hall
190 North Oval Mall
Columbus, OH 43210-1358

Gail Gunderson
Associate Director
The Ohio State University
Office of Human Resources
1590 North High Street, #300
Columbus Ohio 43201

Re: Retaliation Complaint and Misconduct in Research Allegations Complaint

Dear Provost Alutto and Director Gunderson:

I request a formal investigation into the College of Pharmacy's retaliatory investigation of Professor Sheryl L. Szeinbach for her support of my Title VII complaint against the Ohio State University. As you know, my Title VII complaint includes allegations against the Dean of the College of Pharmacy, Professor Robert W. Brueggemeier ("Dean Brueggemeier") and Associate Professor Rajesh Balkrishnan ("Associate Professor Balkrishnan").

During the University's initial investigation into my Title VII complaint, Professor Szeinbach emerged as a key witness in my case. Unfortunately this appears to have caused Dean Brueggemeier and Associate Professor Balkrishnan to mount a campaign to discredit Professor Szeinbach. For example, Associate Professor Balkrishnan accused Professor Szeinbach of improperly citing her own previously published text and data in new publications.

The College of Pharmacy's investigation into Professor Szeinbach is retaliatory because the college decided not to investigate similarly situated faculty. For example, Associate Professor Balkrishnan republished text and data without citation to his prior publications. Associate Professor Balkrishnan is the primary and corresponding author for an article published in Clinical Therapeutics (Balkrishnan R, Arondekar BV, Camacho FT, Shenolikar RA, Horblyuk R and Anderson RT. Comparisons of rosiglitazone versus pioglitazone monotherapy introduction and associated health care utilization in Medicaid-enrolled patients with type 2 diabetes mellitus. Clinical Therapeutics. 2007;29:1306-1315) that does not reference the following 2004 paper: Balkrishnan R, Rajagopalan R, Shenolikar RA, Camacho FT, Whitmire JT and Anderson RT. Healthcare costs and prescription adherence with

the following 2003 paper: Brueggemeier RW, Richards JA and Petrel TA. Aromatase and cyclooxygenases: enzymes in breast cancer. Journal of Steroid Biochemistry & Molecular Biology. 2003;86:501-507.

Dean Brueggemeier reuses many of the same research results (i.e. figures) from the 2005 article in a subsequent 2006 article, again without any reference to the prior publication. For instance, figures 1, 2, 3 and 8 from the 2005 article appear without citation in the following 2006 article: Diaz-Cruz ES. and Brueggemeier RW. Interrelationships between cyclooxygenases and aromatase: unraveling the relevance of cyclooxygenase inhibitors in breast cancer. Anti-Cancer Agents in Medicinal Chemistry. 2006;6:221-232.

Dean Brueggemeier also re-used text and research data in the article "Richards JA and Brueggemeier RW. Prostaglandin E2 regulates aromatase activity and expression in human adipose stromal cells via two distinct receptor subtypes. Journal of Clinical Endocrinology & Metabolism. 2003;88(6):2810-2816" without citation to the prior publication " Brueggemeier RW, Richards JA, Joomprabutra S, Bhat AS and Whetstone JL. Molecular pharmacology of aromatase and its regulation by endogenous and exogenous agents. Journal of Steroid Biochemistry & Molecular Biology. 2001;79:75-84."

Two other publications with Dean Brueggemeier as first or senior author were also not referenced while several figures and tables reappeared in the 2005 and 2003 articles discussed above. These publications are: Richards JA, Petrel TA. and Brueggemeier RW. Signaling pathways regulating aromatase and cyclooxygenases in normal and malignant breast cells. Journal of Steroid Biochemistry & Molecular Biology. 2002;80:203-212 and Brueggemeier RW, Quinn AL, Parrett ML, Joarder FS, Harris RE, and Robertson FM. Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. Cancer Letters. 1999;140:27-35.

At least two pairs of Dean Brueggemeier's articles were submitted and published at about the same time. The first pair with substantial overlap in data is the above 2005 article and the article: Diaz-Cruz ES, Shapiro CL and Brueggemeier RW. Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells. Journal of Clinical Endocrinology & Metabolism. 2005;90:2563-2570. The second pair is formed by the two listed articles published in 2003.

The fact that the college turns a blind eye to Dean Brueggemeier and Associate Professor Balkrishnan's actions while actively investigating Professor Szelnbach provides further evidence of its retaliation against me for my Title VII complaint. As a result, I request the Office of Human Resources investigate this retaliation.

Moreover, since the above-described potential misconduct by Dean Brueggemeier and Associate Professor Balkrishnan appears to violate the University Policy Concerning Misconduct in Research or Scholarly Activities, I request an investigation of this potential misconduct.

I regret I am forced to bring these issues to your attention in this manner. But, the retaliatory activities of Dean Brueggemeier and Associate Professor Balkrishnan are spreading well beyond just me. Specifically, their actions are now impacting the College of Pharmacy's faculty, students, and reputation. Therefore, I thank you in advance for your assistance in addressing these issues.

Sincerely,

Enrique C. Seoane-Vazquez

EXHIBIT 14

Office of Research Compliance

300 Research Foundation
1960 Kenny Road
Columbus, OH 43210

Phone (614) 292-4284
Fax (614) 292-5913

CONFIDENTIAL

June 11, 2008

VIA COURIER DELIVERY

Dr. Enrique Seoane-Vazquez
Assistant Professor
College of Pharmacy
129B Parks Hall
500 W Twelfth Avenue
CAMPUS

Dear Dr. Seoane-Vazquez:

In reference to my letter dated May 29, 2008, I have enclosed a copy of the Final Report of the Committee of Initial Inquiry. As per the University's Policy on Misconduct in Research or Scholarly Activities, you may file a written appeal to the Vice President for Research, Dr. Robert McGrath, within one week of receiving this final report.

Sincerely,

Jennifer S. Moseley

Jennifer S. Moseley, Ph.D.
Research Integrity Officer
Office of Research Compliance

Enclosure

Cc: Office of Research Compliance
R. Brueggemeier

Final Report of the Committee of Initial Inquiry Concerning Allegations of Misconduct in Research or Scholarly Activity

June 4, 2008

This Committee of Initial Inquiry was formed in March 2008, to review allegations of research misconduct made against University Professor Robert W. Brueggemeier, by University Assistant Professor Enrique C. Seoane-Vazquez. The allegations concerned research reviewed in eight (8) published research reports co-authored by Dr. Brueggemeier.

Meetings and Discussions of the Committee:

The committee first met on April 2, 2008. The following documents related to the allegation were provided:

- The University Research Committee Interim Policy and Procedures Concerning Misconduct in Research or Scholarly Activities (the Policy)
- Letter from Dr. Seoane-Vazquez to Dr. Alutto, Executive Vice President and Provost and Ms. Gunderson, Associate Director of Human Resources, alleging research misconduct in the eight (8) publications co-authored by Dr. Brueggemeier, January 18, 2008.
- Letter from Dr. Alutto to Dr. Seoane-Vazquez informing him of the results of the investigation into his allegation against Dr. Brueggemeier, February 5, 2008.
- Letter from Dr. Seoane-Vazquez to Dr. Alutto, requesting an additional investigation into the allegation made against Dr. Brueggemeier, February 11, 2008.
- Letter from Dr. Carole Anderson, Vice Provost, to Dr. Robert McGrath, Senior Vice President for Research, regarding the results of the Preliminary Review, which included the recommendation to initiate an Initial Inquiry, March 7, 2008.
- Letter from Dr. Robert McGrath to Dr. Jennifer Moseley, Research Integrity Officer, authorizing her to establish a Committee of Initial Inquiry, March 11, 2008.
- Memo from Dr. Brueggemeier to the Committee of Initial Inquiry, discussing the publications contained within the allegation, March 10, 2008.
- Letter from Dr. H. B. Kostenbauder to the Office of Research Integrity, alleging research misconduct by Dr. Brueggemeier in eight (8) publications, March 26, 2008.
- R.W. Brueggemeier, A.L. Quinn, M.L. Parrett, F.S. Joarder, R.E. Harris, and F.M. Robertson, Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. Cancer Letters, 140, 27-35 (1999).
- J.A. Richards, T.A. Petrel, and R.W. Brueggemeier, Signaling pathways regulating aromatase and cyclooxygenases in normal and malignant breast cells. J. Steroid Biochem. Molec. Biol., 80, 203-212 (2002).

- J.A. Richards and R.W. Brueggemeier, Prostaglandin E2 regulates aromatase activity and expression in human adipose stromal cells via two distinct receptor subtypes. J. Clin. Endocrinol. Metab., 88, 2810-2816 (2003).

- E.S. Diaz-Cruz, C.L. Shapiro, and R.W. Brueggemeier, Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells. J. Clin. Endocrinol. Metab., 90, 2563-2570 (2005).

- R.W. Brueggemeier, J.A. Richards, S. Joomprabutra, A.S. Bhat, and J.L. Whetstone. Molecular pharmacology of aromatase and its regulation by endogenous and exogenous agents. J. Steroid Biochem. Molec. Biol., 79, 75-84 (2001).

- R.W. Brueggemeier, J.A. Richards and T.A. Petrel, Aromatase and Cyclooxygenase: Enzymes in breast cancer. J. Steroid Biochem. Molec. Biol., 86, 501-507 (2003).

- R.W. Brueggemeier, E.S. Diaz-Cruz, P.-K. Li, Y. Sugimoto, Y.C. Lin, C.L. Shapiro, Translational studies on aromatase, cyclooxygenases, and enzyme inhibitors in breast cancer. J. Steroid Biochem. Molec. Biol., 95, 129-136 (2005).

- E.S. Diaz-Cruz and R.W. Brueggemeier, Interrelationships between cyclooxygenases and aromatase: Unraveling the relevance of cyclooxygenase inhibitors in breast cancer. Anti-Cancer Agents Med. Chem., 6, 221-232 (2006).

After reviewing the documents listed above, the Committee met again on May 13, 2008, to discuss the allegations and related information. The Committee discussed the case at length and arrived at the conclusions described below.

Conclusion of the Committee:

The Committee of Initial Inquiry evaluated the January 18, 2008 letter from Professor Seoane-Vazquez to Provost Joseph Alutto that contained charges of research misconduct by Dean Robert Brueggemeier. Committee members identified five charges contained in paragraphs four through eight of this letter. Each charge was evaluated individually by committee members and were discussed when the committee met as a whole.

1. "Similarly, Dean Brueggemeier is the primary and corresponding author for an article published in the journal of Steroid Biochemistry and Molecular Biology (Translational studies on aromatase, cyclooxygenases, and enzyme inhibitors in breast cancer. 2005; 95: 129-136) that does not reference the following 2003 paper: Brueggemeier RW, Richards JA and Petrel TA. Aromatase and cyclooxygenases: enzymes in breast cancer. Journal of Steroid Biochemistry and Molecular Biology. 2003; 86: 501-507."

Committee members evaluated the nature of these two papers as "proceedings" rather than primary literature (articles presenting primary data). These two proceedings articles were invited manuscripts based on presentations to international research conferences and were clearly marked as proceedings and introduced as proceedings from a meeting by the editors at the beginning of each journal issue. Committee members recognized that such presentations contain previously published and unpublished research data. The former article provided a context and reported recent yet to be published discoveries. The proceedings manuscripts are invited manuscripts and summarize the oral meeting presentation. This was the case with both of these manuscripts which shared data. The

committee carefully examined each article and the data questioned by Professor Seoane-Vazquez was found to be referenced to the peer-reviewed primary literature (articles that are unsolicited and neither proceedings nor reviews). This work was published in Cancer Letters 140 27-35 (1999) and properly quoted in both proceedings. The committee discussed as to whether or not it was best practice to provide quotes to the primary literature or to other proceedings and reviews or to both. The committee believes and observes as common practice in the field of scientific publication to prefer quotation to the primary literature. This is based on the necessity of the authors revealing the most detailed description of how the experiments were designed, performed, and evaluated. This is found within the primary source and is less frequently provided in such detail in reviews or proceedings. Thus the committee found no evidence to indicate that research misconduct may have occurred. The committee noted that this information, including the citations in the proceedings to the primary literature and the primary 1999 Cancer publication, was either available or known to Professor Seoane-Vazquez.

2. "Dean Brueggemeier also reuses many of the same research results (i.e. figures) from the 2005 article in a subsequent 2006 article, again without any reference to the prior publication. For instance, figure 1, 2, 3, and 8 from the 2005 article appear without citation in the following 2006 article: Diaz-Cruz ES. and Brueggemeier RW. Interrelationships between cyclooxygenases and aromatase: unraveling the relevance of cyclooxygenase inhibitors in breast cancer. Anti-Cancer Agents Medicinal Chemistry. 2006; 221-232."

After careful examination of the two articles (JSBMB 95 129-136 (2005) and ACAMC 6 221-232 (2006)) the committee identified the JSBBM manuscript as a proceedings and the ACAMC article as a review. The figures found in the articles were properly quoted from the primary literature (Journal of Clinical Endocrinology and Metabolism 90 2563-2570 (2005)). We find that the analysis described in the preceding complaint (1.) is directly applicable to this charge. Thus the committee found no evidence to indicate that research misconduct may have occurred. The committee noted that this information including the citations in the proceeding and the review to the primary literature and the primary 2005 JCEM article was known to Professor Seoane-Vazquez.

3. "Dean Brueggemeier also re-used text and research data in the article 'Richards JA and Brueggemeier RW. Prostaglandin E2 regulates aromatase activity and expression in human adipose stromal cells via two distinct receptor subtypes. Journal of Clinical Endocrinology and Metabolism. 2003; 88 (6): 2810-2816' without citation to the prior publication 'Bruggemeier RW, Richards JA, Joomprabutra S, Bhat AS and Whetstone JL. Molecular pharmacology of aromatase and its regulation by endogenous and exogenous agents. Journal of Steroid Biochemistry and Molecular Biology. 2001;79: 75-84.

The committee carefully examined both articles and identified the 2001 JSBMB as a proceeding and the 2003 JCEM as an unsolicited peer-reviewed entry in the primary literature. The committee disagrees with Professor Seoane-Vazquez's statement that JSBMB 2001 is not quoted in the JCEM 2003 article. JSBMB 2001 can be found as reference 41 in JCEM 2003. As previously stated, the committee finds it common practice that proceedings often contain unpublished data that is subsequently published in the primary literature. This is the case here, where the JCEM 2003 article contains enhancements to the preliminary data that were not in the proceedings including statistical analysis, enhanced methods and discussion. Thus the committee found no evidence to indicate that research misconduct may have occurred. The committee noted that the information of the nature of these two articles and the quotation of the proceedings in the JCEM 2003 article was available to Professor Seoane-Vazquez.

4. "Two other publications with Dean Brueggemeier as first or senior author were also not referenced while several figures and tables reappeared in the 2005 and 2003 articles discussed above. These publications are Richards JA, Petrel TA and Brueggemeier RW. Signaling pathways regulating aromatase and cyclooxygenases in normal and malignant breast cells. Journal of Steroid Biochemistry and Molecular Biology. 2002; 80: 203-212 and Brueggemeier RW, Quinn AL, Parrett ML,Joarder FS, Harris RE and Robertson FM. Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. Cancer Letters 1999; 140: 27-35."

The complaint is unclear as to which two articles from 2003 and 2005 contain materials from Cancer Letters 140 27-35 (1999) and JSBMB 80 203-212 (2002). Both the 1999 and 2002 articles contain primary data. Due to this ambiguity, we examined all articles that were included in the complaint and published in 2003 and 2005 for overlapping and unquoted tables and figures. The specific articles evaluated included JSBMB 86 501-507 (2003), JSBMB 95 129-136 (2005), JCEM 88 2810-2816 (2003), and JCEM 90 2563-2570 (2005). The first two articles are clearly marked as proceedings while the latter two articles are unsolicited peer-reviewed primary data.

The proceedings. JSBMB 86 501-507 (2003) contains data from Cancer Letters (1999) and JSBMB (2002) and both are correctly quoted by references 17 and 33. JSBMB 95 129-136 (2005) contains data from Cancer Letters (1999) this properly quoted in reference 18. JSBMB (2003) contains no data from JSBMB (2002) and is not quoted.

The primary data. JCEM (2003) contains no data from JSBMB (2002) although it is quoted in the *Methods* section as reference 43. JCEM (2003) contains no information from Cancer Letters (1999), this manuscript is not quoted. Finally, JCEM (2005) has no data from either Cancer Letters (1999) or JSBMB (2002). Cancer Letters (1999) is mentioned in the introduction and is reference 15 while JSBMB (2002) is neither discussed nor quoted.

We are unable to find any evidence for unreferenced primary data from any of these two articles in any of the four 2003 or 2005 manuscripts. Thus the committee found no evidence to indicate that research misconduct may have occurred. The committee noted that the information of the nature of these manuscripts and their quotation was available to Professor Seoane-Vazquez.

5. "At least two pair of Dean Brueggemeier's articles were submitted and published at about the same time. The first pair with substantial overlap in data is the above 2005 article and the article: Diaz-Cruz ES, Shapiro CL, and Brueggemeier RW. Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells. Journal of Clinical Endocrinology & Metabolism. 2005; 90: 2563-2570. The second pair is formed by the two listed articles published in 2003."

The 2003 articles in question are JSBMB 86 501-507 (2003) and JCEM 88 2810-2816 (2003). The former article is a proceeding while the latter article is non-solicited peer-reviewed primary data. The 2005 articles are JCEM 90 2563-2570 (2005) and JSBMB 95 129-136 (2005). The former article is non-solicited peer-reviewed primary data while the latter article is a proceeding.

The two 2003 articles do not quote each other. JCEM (2003) was submitted in September 2002, accepted for publication in March 2003, and published in June 2003. The JSBMB (2003) was submitted after the primary data article was submitted and was published in September 2003. The JCEM (2003) was not published when the proceedings were submitted and could not be quoted (page 8 of Dean Brueggemeier's response dated March 10, 2008).

[illegible] The JCEM (2005) article is quoted in the

which the citations were available made this latter quotation not possible (page 8 of Dean Brueggemeier's response dated March 10, 2008).

The committee found no evidence to indicate that research misconduct may have occurred.

In summary, after extensive review of the January 18, 2008 letter of complaint and all materials subsequently provided to the committee, we find insufficient evidence to support charges of research misconduct by Dean Robert Brueggemeier.

Based on the specific information used to prepare these charges and the omission of obvious information that would lead to their dismissal, the committee finds that Professor Enrique C. Seoane-Vazquez provided frivolous charges in his January 18, 2008 letter and in doing so abused the procedures set forth in the University Research Committee Interim Policy and Procedures Concerning Misconduct in Research or Scholarly Activities.

The February 11, 2008 from Professor Seoane-Vazquez adds several additional charges to those we have considered in his January 18, 2008 letter. These include:

1a. self plagiarism: extensive text recycling
3. violation of journal policies on submission and publication
4. copyright infringement.

The committee believes items 1b, 1c, and 2 have been evaluated based on the complaints provided in the January 18, 2008 letter and provided no new evidence that research misconduct may have occurred.

1a. Various portions of text can be observed to be essentially duplicated in several articles authored by Dean Brueggemeier and colleagues. These duplications are primarily found between articles containing primary data and proceedings most commonly in *Introductions* and *Methods* sections. These types of duplications are commonly found in review or proceedings articles. They represent a lack of crafting an independent argument for each scientific communication. In similar cases, Committees of Initial Inquiry have found that more extensive self-quotations have not amounted to charges of research misconduct. Thus, the committee finds insufficient evidence to support a finding that research misconduct may have occurred.

3 and 4. Violations of journal policies on submission and publication and copyright are issues that need to be resolved between authors and journal editors. The agreements that are struck between journal editors and the authors concerned should be dealt with exclusively by these parties. In previous cases of research misconduct, these issues have been left to the journal editors and authors by the Committee of Initial Inquiry. We have evidence in the present case that these issues have been raised with journal editors. The editors of the Journal of Clinical Endocrinology and Metabolism appear to have investigated the complaint and have reached a resolution with Dean Brueggemeier. On the other hand, contact with Professor Dr. Manfred Schwab, Editor of Cancer Letters, has not resulted in the initiation of an appropriate investigation. We urge Professor Dr. Schwab to initiate and complete a thorough and confidential investigation in a timely fashion. Based on communications provided to the Committee of Initial Investigation, we believe that Professor Dr. Schwab has shared privileged information with Emeritus Professor Kostenbauder of Columbus, Ohio regarding Dean Brueggemeier's submitted publications. Both journal editors and reviewers commonly are responsible for keeping information regarding submitted manuscripts confidential. The Committee suggests that Professor Dr. Schwab should discontinue communicating the status of submitted manuscripts to outside individuals who are not directly concerned with manuscript submission.

Appendix

Complainant:

Dr. Enrique C. Seoane-Vazquez, Assistant Professor, College of Pharmacy

Respondent:

Dr. Robert W. Brueggemeier, Professor and Dean, College of Pharmacy

Committee Members:

Dr. Charles L. Brooks (Chair), Professor, College of Veterinary Medicine
Dr. Dale D. Vandre, Associate Professor, College of Medicine
Dr. Matthew S. Platz, Distinguished University Professor and Interim Dean, College of Mathematical and Physical Sciences

Dr. A. Douglas Kinghorn, Jack L. Beal Professor and Chair, College of Pharmacy (non-voting consultant)

Misconduct Coordinator:

Jennifer S. Moseley, Ph.D.

Correspondence:

Letter from Dr. Seoane-Vazquez to Dr. Alutto, Executive Vice President and Provost and Ms. Gunderson, Associate Director of Human Resources, alleging research misconduct in the eight (8) publications co-authored by Dr. Brueggemeier, January 18, 2008.

Letter from Dr. Alutto to Dr. Seoane-Vazquez informing him of the results of the investigation into his allegation against Dr. Brueggemeier, February 5, 2008.

Letter from Dr. Seoane-Vazquez to Dr. Alutto, requesting an additional investigation in to the allegation made against Dr. Brueggemeier, February 11, 2008.

Letter from Dr. Carole Anderson, Vice Provost, to Dr. Robert McGrath, Senior Vice President for Research, regarding the results of the Preliminary Review, which included the recommendation to initiate an Initial Inquiry, March 7, 2008.

Letter from Dr. Robert McGrath to Dr. Jennifer Moseley, Research Integrity Officer, authorizing her to establish a Committee of Initial Inquiry, March 11, 2008.

Letters to the designated Committee of Initial Inquiry members from Dr. Robert McGrath, March 13, 2008.

Letter from Dr. Charles L. Brooks, Chair of the Committee of Initial Inquiry, to Dr. Robert Brueggemeier, informing him of the composition of the Committee of Initial Inquiry, April 8, 2008.

Memo from Dr. Robert Brueggemeier to Drs. Carole Anderson and Todd Guttman, informing them of his recent communication with the Journal of Clinical Endocrinology and Metabolism, April 7, 2008.

E-mail from Dr. Robert Brueggemeier to Dr. Manfred Schwab, Editor in Chief of Cancer Letters, requesting that Dr. Schwab provide him with the ability to respond to an allegation of research misconduct, May 12, 2008.

EXHIBIT 15

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June 24, 2008

Robert T. McGrath, Ph. D.
Senior Vice President For Research
The Ohio State University
208 Bricker Hall
190 North Oval Mall
Columbus, Ohio 43210

RE: Written Response To Final Report Of the Committee of Initial Inquiry Concerning Allegations of Misconduct In Research or Scholarly Activity.

Dear: Dr. McGrath:

Thank you for providing my client, Dr. Enrique Seoane-Vazquez, the *Final Report Of the Committee of Initial Inquiry Concerning Allegations of Misconduct In Research or Scholarly Activity* detailing the Committee's findings regarding allegations of research misconduct by Dean Robert W. Brueggemeier ("Final Report"). Since I currently represent both Dr. Enrique Seoane-Vazquez and Dr. Sheryl Szeinbach with regard to their complaints against The Ohio State University ("OSU"), Drs. Seoane-Vazquez and Szeinbach requested I draft this appeal. Therefore, per OSU's Policy on Misconduct, this document is Dr. Seoane-Vazquez's written appeal of the Final Report.

In reviewing the Final Report, at least six conflicts with the *University Research Committee Interim Policy and Procedures Concerning Misconduct in Research of Scholarly Activities* ("Misconduct Policy") were identified. I detail those conflicts below.

1. <u>Violation of Misconduct Policy §V(E)'s Conflicts Of Interest Provisions</u>

The Final Report should be deemed void because the Committee's membership violated Misconduct Policy §V(E). This provision states, "all persons involved [in the Committee] shall be vigilant to prevent any real or perceived conflict of interest." As a result, persons who engaged in "co-authorship of work within the recent past with . . . individuals . . . involved in the alleged misconduct" should have been barred from the Committee. *Id.* Therefore, since both Drs. Kinghorn and Vandre co-authored publications with Dean Brueggemeier in the recent past, they should not have been permitted to participate in the Committee. *See, Balunas, Marcy, J; Su, Bin; Landini, Serena; Brueggemeier, Robert, W; Kinghorn, A Douglas. Interference by naturally*

occurring fatty acids in a noncellular enzyme-based aromatase bioassay. Journal of natural products. 69 (2006) 700-3; Brueggemeier, RW, Bhat, AS, Lovely, CJ, Coughenour, HD, Joomprabutra, S, Weitzel, DH, Vandre, DD, Yusif, F, Burak, WE, Journal of Steroid Biochemistry & Molecular Biology 78 (2001) 145-156.

Misconduct Policy §V(E) also bars individuals with "subordinate job relationships" with subjects of misconduct investigations from participating in "any investigative or decisional" role in a misconduct investigation. In this case, Dr. Kinghorn is a "subordinate" of Dean Brueggemeier.

OSU's decision to exclude recent co-authors and subordinates from involvement in misconduct investigations makes perfect sense. This is because committee members may have a strong incentive to clear the reputations of their co-authors in order to avoid any negative splash back onto themselves. Moreover, subordinates naturally fear the repercussions of finding their superiors committed scientific misconduct.

Therefore, the Final Report should be rendered void and the Committee should reform itself in accordance with Misconduct Policy §V(E). Moreover, to remove the taint caused by the violation of Misconduct Policy §V(E), OSU should appoint an entirely new Committee. Otherwise the Committee cannot meet its mandate to be "vigilant to prevent any real or perceived conflict of interest." *Misconduct Policy §(V)(E).*

2. **Evidence Presented To Committee Established Dean Brueggemeier's Violation of Misconduct Policy III(A)(2).**

The Final Report advances three arguments that excuse Dean Brueggemeier violation of the Misconduct Policy. But, none of these arguments are based on "commonly accepted practices" within the scientific community. First, the Committee alleges Dean Brueggemeier did not engage in redundant publications because many of the suspect publications were "proceedings." *See e.g., Final Report at pgs. 2-3.*

In support of this position, the Committee stated, it is "common practice that proceedings often contain unpublished data that is subsequently published in the primary literature." *Final Report p. 3.* The Committee's "proceeding" defense of Dean Brueggemeier, however, conflicts with commonly accepted publication guidelines for "proceedings." For example, COPE guidelines state, "[p]revious publication of an abstract during the proceedings of meetings does not preclude subsequent submission for publication, but full disclosure should be made at the time of submission." *Committee on Publication Ethics (COPE Guidelines On Good Publication Practice (2003), pgs 70-71; See also, The American Chemical Society's Ethical Guidelines to Publication of Chemical Research (2006), p. 3* ("ACS Publication Guidelines") (permitting the republication of "previously published brief preliminary account (a 'communication' or 'letter') of the same work . . . [provided] the editor should be made aware of the earlier communication, and the preliminary communication should be cited in the manuscript."); *The American Chemical Society's Ethical Guidelines to Publication of Chemical*

Research (2006); Ethics and scientific publication Dale J. Benos, Jorge Fabres, John Farmer, Jessica P. Gutierrez, Kristin Hennessy, David Kosek, Joo Hyoung Lee, Dragos Olteanu, Tara Russell, Faheem Shaikh, and Kai Wang, Adv Physiol Educ 29: 59–74, 2005; p. 64 (noting, the American Physiological Society Ethical Policy stating, ". . . journals of the APS only accept research papers that are original work, no part of which has been submitted for publication elsewhere except as a brief (i.e., 400 words) abstract.").

In fact, commentator who addressed the interplay between COPE guidelines and "proceedings" noted:

> Many authors are under the mistaken impression that publication of a manuscript in a society proceedings as a full manuscript does not preclude publication in substantially similar forms in other journals. The COPE guidelines explicitly note that republication is appropriate only when the proceedings contains the abstract alone (typically a few hundred words). Thus, publication of full multiple-page manuscripts in a society proceedings constitutes a publication in its own right, often with copyright transference to another party. *J Bone Joint Surg Am. 2006;88:2323-2325. doi:10.2106/JBJS.F.01154 Richard A. Brand, Joshua J. Jacobs and James D. Heckman, pg. 2324 (emphasis added).*

In this case, Dean Brueggemeier's "proceedings" greatly exceeded the "few hundred words" typically associated with COPE's guidelines on republishing proceeding materials. Moreover, Dean Brueggemeier's "proceedings" were peer reviewed. As a result, commentators suggest the Committee's "proceeding" defense of Dean Brueggemeier fails. For example, one group of commentators states:

> Most papers in conference proceedings are not subjected to the stringent peer-review process of the IEEE. Publication in such proceedings generally does not preclude submission to and publication in an IEEE peer-reviewed publication, such as the *Magazine*. Furthermore, what appears in most conference proceedings is usually an abstract, or, at most, a relatively short summary paper. The full paper or feature article submitted to a peer-reviewed IEEE publication typically includes substantially more information. Concerns related to double submission or double publication can arise where papers in a proceedings are subjected to full peer review. These concerns can also arise when the papers in the proceedings are extensive, and, for example, when the proceedings are published in book form (that is, available for purchase as a book by a major publisher, and indexed and cataloged as a hook – as the problem." *An editorial: Plagiarism, Duplicate Publication, and Duplicate Submission: They Are All Wrong! W. Ross Stone Editor-in-Chief, EEE Antennas and Propagation Magazine, Vol. 45, No. 4, August 2003, pages 46-47.*

In this case, however, the Final Report did not address any of the aforementioned issues related to proper "proceeding" publication standards. Had the committee done so, it should have determined Dean Brueggemeier violated Misconduct Policy III(A)(2). In fact, such a determination could have also been reached by comparing Dr. Brueggemeier "proceeding" publications with another proceeding author from the Aromatase 2004 meeting - Dr. Angela Brodie of the University of Maryland.

Dr. Brodie is similar to Dr. Brueggemeier in seniority, and both have published extensively in the aromatase area. Dr. Brodie and Dr. Brueggemeier both attended and presented their work at the Aromatase 2004 meeting held on September 6-8, 2004. They both subsequently published their presentations in the proceeding that appeared in *J. Steroidal Biochemical & Molecular Biology (JSBMB). Brodie A, Jelovac D, Sabnis G, Long B, Macedo L, Goloubeva O. Model systems: mechanisms involved in the loss of sensitivity to letrozole. J Steroid Biochem Mol Biol. 2005 May;95(1-5):41-8.* After the meeting, both submitted primary research articles that contained materials presented at the Aromatase 2004 meeting - Dr. Brodie on November 11, 2004, and Dr. Brueggemeier on October 13, 2004.

Dr. Brodie's paper appeared in the May 2005 issue of Cancer Research. *See, Sabnis GJ, Jelovac D, Long B, Brodie A. The role of growth factor receptor pathways in human breast cancer cells adapted to long-term estrogen deprivation. Cancer Res. 2005 May 1;65(9):3903-10.* Dr. Brueggemeier's paper appeared in the May 2005 issue of J Clinical Endocrinology and Metabolism. *See, Diaz-Cruz ES, Shapiro CL, Brueggemeier RW. Cyclooxygenase Inhibitors Suppress Aromatase Expression and Activity in Breast Cancer Cells. J Clin Endocrinol Metab 90: 2563–2570, 2005.* Nevertheless, while Dr. Brodie's publications followed publication ethics, Dr. Brueggemeier's publications did not as evidenced by the table below:

Publication ethics & expectation of journal editors and readership	Dr. Brodie's publications	Dr. Brueggemeier's publications
Data in primary research articles should be original and not published in other forms of mass media*	• Dr. Brodie's Cancer Research paper contained 6 figures. Parts of the data in Fig. 3 (~50%) contained similar but not identical materials as in her Proceeding publication in JSBMB. • Dr. Brodie's two papers did not contain paragraphs of identical text.	• As detailed in Dr. Seoane-Vazquez's letter to Dr. Alutto, dated February 11 2008, more than 50% of the results and text in Dr. Brueggemeier's JCEM publication were materials that were published in the Proceeding in JSBMB.
Editors and readers expect to be informed of duplicated materials	• Dr. Brodie's Cancer Research (primary research) paper indicated in the footnote that the work was partly presented at the Aromatase 2004 meeting.	• Dr. Bruggemeier did not indicate in his JCEM (primary research) paper that he previously presented the related work at the Aromatase 2004 meeting. Hence, the JCEM

		Editor and readers of his JCEM did not know of the duplicated materials. • The Editor of JCEM, Dr. Ladenson, in his 3/18/08 letter wrote to Dr. Brueggemeier, demanded apologies from Dr. Brueggemeier to JCEM and the Endocrine Society for publishing "minimally different publications" in JCEM. This evidences Dr. Brueggemeier did not inform the JCEM editors of the duplicated materials/publications.
Proper citation	• Dr. Brodie properly cited her presentation at the Aromatase 2004 meeting.	• As detailed in Dr. Seoane-Vazquez's 2/11/08 letter to Dr. Alutto, Dr. Brueggemeier improperly cited the JSBMB paper. None of the citations (3 times) properly informed the editors or readers that the materials in the primary research article had been presented at the Aromatase 2004 meeting and would be published in a widely distributed journal. • One of the citations of the JSBMB paper in the JCEM paper was for the "method of aromatase enzyme activity measurement". This is incorrect for at least three reasons. (1) Dr. Brueggemeier first published the same method before 1999. (2) Dr. Brueggemeier submitted the JCEM paper after he submitted the JSBMB (hence, JSBMB cannot be the "previous" publication). Dr. Brueggemeier submitted the first draft of the JSBMB paper 2.5 months after submitted the first version of his JCEM paper, 4 days after he

		submitted the revised version of the JCEM paper and two days after assigning the copying right to JCEM. *See, Dr. Brueggemeier' May 11, 2008 letter to to Editor of Cancer Letters Manfred Schwab, p.9.* (3) Dr. Brueggemeier only referred to a method of analysis, and not to the results.
Copyright	• Dr. Brodie used the data in her JSBMB as preliminary data (see below). As such, she did not violate the copyright.	• The data in Dr. Brueggemeier's JSBMB paper does not qualify as preliminary data (see below). • Dr. Brueggemeier assigned the copyrights to the duplicated materials to two different journals, as stated in his Letter to the Editor of Cancer Letters (p.9). The copyright assignment to JCEM was on 1/5/08, or two days after Dr. Brueggemeier submitted the first version of his JSBMB paper.
Overall	Dr. Brodie adhered to publication ethics • By clearly informing the reader that some of the data of her Cancer Research article had been presented previously. • By re-using only a small amount of the data in her JSBMB paper and by supplementing the data with additional data, the data appearing in the JSBMB paper qualifies as preliminary data, as meant by publications of meeting proceedings, according to COPE Guidelines.	Dr. Brueggemeier did not adhere to publication ethics • He failed to indicate the presentation of the majority of the results in the JCEM at the Aromatase meeting (and therefore the JSBMB paper). • His massive duplication without meaningful expansion takes away the argument that his datasets appearing in the JSBMB paper constitute preliminary data upon which the JCEM paper was based, as argued by the Committee of Initial Inquiry. • The massive duplication also takes away the argument that the JSBMB paper was a review since reviews should review more than a single original piece of work, and should bring new perspective. A duplicate

		publication does neither.

What should Dean Brueggemeier have done differently to avoid violating the Misconduct Policy? For one thing, he should have put the various editors on notice that he was engaging the "dual [sic] publication" that JCEM later determined he engaged in. *See, March 18, 2008 letter from Dr. Paul W. Ladenson to Dr. Brueggemeier.* One commentator explains that such notice should have appeared:

> in a cover letter to the editor . . . [stating] the material has appeared in an abstract published in a previous society proceedings or when, for any reason, the material is under consideration or in press at another journal. *J Bone Joint Surg Am. 2006;88:2323-2325. doi:10.2106/JBJS.F.01154 Richard A. Brand, Joshua J. Jacobs and James D. Heckman, pg. 2324.*

This is because "[f]ull disclosure requires notation of those elements of the manuscript that are redundant and of the reasons for republishing them. *J Bone Joint Surg Am. 2006;88:2323-2325. doi:10.2106/JBJS.F.01154 Richard A. Brand, Joshua J. Jacobs and James D. Heckman, pg. 2324.*

The sending of such notices directly squares with the scientific publication mandates that should have guided Dr. Brueggemeier and the Committee's Misconduct Policy evaluation. For example, COPE guidelines state authors of previously published materials such as proceedings, "should disclose details of related papers . . . and similar papers in press." *Committee on Publication Ethics (COPE), Guidelines On Good Publication Practice (2003), p. 71.*

Similarly, the World Association of Medical Editors (WAME) guidelines note, "[p]revious publication of an abstract during the proceedings of meetings (in print or electronically) does not preclude subsequent submission for publication, but full disclosure should be made at the time of submission." http://www.wame.org/resources/publication-ethics-policies-for-medical-journals (15 of 26) 6/8/2008 12:13:36 PM (emphasis added). This disclosure is also required by ICMJE rules that state:

> "[w]hen submitting a paper, the author must always make a full statement to the editor about all submissions and previous reports (including meeting presentations and posting of results in registries) that might be regarded as redundant or duplicate publication of the same or very similar work. The author must alert the editor if the manuscript includes subjects about which the authors have published a previous report or have submitted a related report to another publication. Any such report must be referred to and referenced in the new paper. Copies of such material should be included with the submitted paper to help the editor decide how to handle the matter. If redundant or duplicate publication is attempted or occurs without such notification, authors should expect editorial action to be taken. At the least, prompt rejection of the submitted manuscript should be

> expected. If the editor was not aware of the violations and the article has already been published, then a notice of redundant or duplicate publication will probably be published with or without the author's explanation or approval. *ICMJE - Uniform Requirements for Manuscripts Submitted to Biomedical Journals 6/7/2008, (emphasis added).*

In this case, however, it appears the Committee failed to determine if Dean Brueggemeier adhered to any of these guidelines. In fact, the Final Report is completely devoid of any reference to third-party documentary evidence supporting the Committee's findings. Moreover, the Committee's decisions may have been swayed by Dean Brueggemeier's attempts to misconstrue the opinion of third-party experts.

For example, the Final Report references Dean Brueggemeier's May 11, 2008 letter to Cancer Letters. This letter contained a quote from Dr. Miguel Roig. This quote suggesting Dr. Roig would find Dean Brueggemeier's "duplicate publications" acceptable. *See, Dean Brueggemeier's May 11, 2008 letter to Manfred Schwab Editor and Chief of Cancer Letters at p. 8.* Dean Brueggemeier quoted Dr. Roig as stating, "summaries of abstracts of papers that are published in conference proceedings are often subsequently published in expanded form as a journal article." *Id.*

But, Dean Brueggemeier's quotation of Dr. Roig missed the letter and the spirit of the quoted publication - *Avoiding plagiarism, self-plagiarism, and other questionable writing practices: A guide to ethical writing.* This is because Dr. Roig's article strongly suggests Dean Brueggemeier violated ethical writing guidelines. For instance, Dr. Roig qualifies the sentence quoted by Dean Brueggemeier with the following: "[i]n these and other cases where redundant publication is being considered by the author, the editors and the readers of each paper must be made aware that a second published version exists." *Id.* This is because Dr. Roig believes, "[a]uthors who submit a manuscript for publication containing data, reviews, conclusions, etc., that have already been disseminated in some significant manner (e.g., published as an article in another journal, presented at a conference, posted on the internet) must clearly indicate to the editors and readers the nature of the previous dissemination. *Id.*

Simply put, it appears the Committee neglected to properly consult the commonly accepted scientific rules, policies, standards, and commentaries identified above when evaluating Dean Brueggemeier's publication record. In not doing so, the Committee neglected its obligations under §III(A)(2) of the Misconduct Policy.

The Committee's second defense of Dean Brueggemeier's conduct hinges on its finding that Dean Brueggemeier "could not have quoted" his JSBMB 2005 publication in his JCEM 2005 publication because "[t]he dates at which the citations were available made this latter quotation not possible." *Final Report, at pgs. 4-5.* The Committee's defense of Dean Brueggemeier, however, is unsupported by commonly accepted "in press" publication standards.

For example, COPE guidelines state authors of previously published materials

such as proceedings, "should disclose details of related papers . . . and similar papers in press." *Committee on Publication Ethics (COPE), Guidelines On Good Publication Practice (2003), pgs 70-71,* (emphasis added). Moreover, ICMJE publication rules state,

> "[w]hen submitting a paper, the author . . . must alert the editor if the manuscript includes subjects about which the authors have published a previous report or have submitted a related report to another publication. *ICMJE - Uniform Requirements for Manuscripts Submitted to Biomedical Journals 6/7/2008, (emphasis added).*

In addition, *The American Chemical Society's Ethical Guidelines to Publication of Chemical Research (2006* ("ACS Publication Guidelines") explains that, [a]uthors are expected . . . [to] inform the editor of related manuscripts that the author has under editorial consideration or in press." p.3(emphasis added). *See also, http://www.wame.org /resources/publication-ethics-policies-for-medical-journals (14 of 26), 6/8/2008 12:13:36 PM* ("[r]epublication of a paper . . . may be acceptable, provided that there is full and prominent disclosure of its original source at the time of submission of the manuscript. At the time of submission, authors should disclose details of related papers they have authored, even if . . . similar papers in press, and any closely related papers previously published or currently under review at another journal." (emphasis added); *JACS Notice to Authors of Papers (2008)*(finding, "[w]hen related work by any of the authors is not available because it is in press (accepted), submitted, or in preparation for submission to *JACS* or another journal, a copy of each related paper should be uploaded as "Review-Only Material" at the time of submission for use by the reviewers and the Editors.")*(emphasis added).*

Why is such disclosure required? Because, "the simultaneous submission of manuscripts to more than one journal is considered unethical as there is both a potential for disagreement over the right to publish among the journals and the possibility of unnecessary duplication of peer review and editing." *Ethics in scientific publication, Laragh Gollogly, Hooman Momen, Rev Saúde Pública 2006;40(N Esp):24-9, page 27.*

In advancing its "in press" defense of Dean Brueggemeier, the Committee unfortunately does more than just propose a defense that conflicts with commonly accepted "in press" publication standards. The Committee also advances a factually flawed argument. This is because the Committee's claim that it was impossible for Dr. Brueggemeier to cite the 2005 JCEM paper in his 2005 JSMBM paper is factually inaccurate. This is because the 2005 JCEM paper was first published online on February 1, 2005 – contrary to Dean Brueggemeier's claim that the JCEM paper was published in May 2005. *See, Dean Brueggemeier's May 11, 2008 letter to Cancer Letters' Editor-in Chief Manfred Schwab, at p. 9.* Therefore, the Committee's "in press" defense of Dean Brueggemeier must be rejected.

The Committee's third defense of Dean Brueggemeier involves its claim that his publications conform to commonly accepted practices regarding duplicate publications. This finding is at odds with the objective conclusions of the editors who evaluated Dean

Brueggemeier's duplicate publications and an NIH-funded website. This website identified Dr. Brueggemeier as the author of multiple duplicate publications. *See, http://discovery.swmed.edu/dejavu.*

The Committee's finding is also at odds with ICMJE guidelines. These guidelines define redundant (or duplicate) publications as "a paper that overlaps substantially with one already published in print or electronic media." *ICMJE - Uniform Requirements for Manuscripts Submitted to Biomedical Journals 6/7/2008.*[1] ICMJE maintains duplicate publications such as those by Dean Brueggemeier are "justifiable . . . provided all of the following conditions are met[:]

1. The authors have received approval from the editors of both journals; the editor concerned with secondary publication must have a photocopy, reprint, or manuscript of the primary version.
2. The priority of the primary publication is respected by a publication interval of at least one week (unless specifically negotiated otherwise by both editors).
3. The paper for secondary publication is intended for a different group of readers; an abbreviated version could be sufficient.
4. The secondary version faithfully reflects the data and interpretations of the primary version.
5. The footnote on the title page of the secondary version informs readers, peers, and documenting agencies that the paper has been published in whole or in part and states the primary reference. A suitable footnote might read: "This article is based on a study first reported in the [title of journal, with full reference]." Permission for such secondary publication should be free of charge.
6. The title of the secondary publication should indicate that it is a secondary publication (complete republication, abridged republication, complete translation, or abridged translation) of a primary publication. *Id. (emphasis added)*

In this case, however, the Committee did not determine if Dean Brueggemeier satisfied these conditions. But, JCEM apparently did. This is because JCEM found Dean Brueggemeier's duplicate publications created "two significant problems not satisfactorily explained by [Dean Brueggemeier's] response." *March 18, 2008 letter from Dr. Paul Laderson to Dean Brueggemeier.* JCEM identified these "two significant problems" as:

> First, you submitted as original work for your 2003 JCEM article data that had been previously published in a minimally different format in the Journal of Steroid Biochemistry & Molecular Biology [December 2001] 79:75-84.

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[1] *See also,* The Journal of Clinical Endocrinology & Metabolism's (JCEM) policy identifies "[m]aterial previously published as "materials published in any form of mass communication." JCEM is the journal that determined Dean Brueggemeier engaged in "dual [sic] publications."

> Second, several figures first published in JCEM in 2005, subsequently appeared with minimal revision in the Journal of Steroid Biochemistry & Molecular Biology [May 2005] 95:129-136 and Anti-Cancer Agents in Medicinal Chemistry [May 2006] 6:221-232. To our knowledge, you failed to seek permission from our journal for use of this copyrighted material. Although the JCEM publication is generally cited in the reference list, it is not denoted in the legend to these figures. *Id.*

As a result, JCEM requested Dean Brueggemeier issue an "apology" to JCEM. *Id.* In addition, JCEM instructed Dean Brueggemeier to provide JCEM with the name of the "academic officer at Ohio State so they can determine it these instances were among those previously investigated by your institution." *Id.*

Various commentators explain why Dean Brueggemeier's redundant publications violate Misconduct Policy III(A)(3). For instance, one group of commentators identified six reasons why redundant publications are "unethical."

> "First, it may infringe international copyright law. Second, duplication of data with additional new data wastes the valuable time of expert peer reviewers. Third, it needlessly expands the already extensive body of published literature. Fourth, it confounds scientific communication by dividing rather than combining closely related data from a single group. Fifth, it may unduly overemphasize the importance of the findings by having them appear more than once. Sixth, it may interfere with subsequent meta-analysis by apparently boosting patient or experimental numbers." *Ethics and scientific publication Dale J. Benos, Jorge Fabres, John Farmer, Jessica P. Gutierrez, Kristin Hennessy, I David Kosek, Joo Hyoung Lee, Dragos Olteanu, Tara Russell, Faheem Shaikh, and Kai Wang, Adv Physiol Educ 29: 59–74, 2005; p. 63.*

These commentators note that "duplicate publication [is] considered misconduct . . . [because it is an] obvious attempt to inflate one's own publication record . . . ." *Id. See also, Ethics in scientific publication, Laragh Gollogly, Hooman Momen, Rev Saúde Pública 2006;40(N Esp):24-9, p. 27* (finding "[r]edundant publications are considered unethical for many reasons: they waste the time of peer reviewers and editors, consume journals' resources and fill pages, increase the work of indexing and abstracting services, distort the academic rewards' system and inflate the scientific literature, all for no benefit other than to the author. Duplicate publications may also infringe on copyright, and contribute to flawed meta-analysis.").

In this case, Dean Brueggemeier's conduct appears to provide evidence of these commentators' concerns regarding the use of duplicate publications to inflate one's publication records. This is because Dean Brueggemeier's 2007 Self-Assessment of his activities at OSU claims he maintained a very high level of scholarly activity by

because at least some of these publications have been judged duplicate publications by their publishers.

The perils of engaging in duplicate publications is something the Committee is well aware of. In fact, one of Committee members' has gone on record detailing the "ethical violations" of duplicative publications. *See, Scientific Integrity Matters (Really Maters) PowerPoint Presentation, Dr. A. Douglas Kinghorn, Jack L. Beal Professor and Editor-in-Chief, Journal of Natural Products, attached as Exhibit 1.* Specifically, Dr. Kinghorn noted duplicative publications create "ethical violations" that often trigger two sanctions: (1) a journal "can forbid the author in question from submitting to the journal for a specified period;" and (2) the matter may be turned over to the institutional Office of Research Integrity of the institution of the lead author, in order to set up an inquiry." *Id.*

In this case, both of the sanctions identified by Dr. Kinghorn occurred. First, the editor of Cancer Letters stated Dean Brueggemeier "would be banned from any publication in journals where [he has] some kind of influence." *March 5, 2008 email from Manfred Schwab to Dr. H.B. Kostenbauder, attached as Exhibit 2.* This is because Dr. Schwab found Dean Brueggemeier's publications "totally unacceptable and . . . by no means . . .] accept[able] by the scientific community. . . .". *Id.* Second, the editors of JCEM asked OSU to investigate Dean Brueggemeier for the "significant problems" created by his duplicate publication in JCEM of his 2003 JCEM publication. *March 18, 2008 letter from Dr. Paul W. Ladenson to Dr. Brueggemeier.*

Nevertheless, the Committee discounted JCEM's determination that Dean Brueggemeier engaged in unauthorized duplicative publication. The Committee did so because it "appear[s] . . . [JCEM] reached a resolution with Dean Brueggemeier." *Final Report, p. 5.* But, the nature of the "agreement" was not revealed. This is an error in judgment because details of this "resolution" could indicate a serious deviation from acceptable practices. Therefore, the "resolution" should be presented to the Committee. This is because such "resolutions" can include sanctions such as, dismissal from societies for a number of years, expulsion, written notification to other professional societies, institutions and/or funding agencies. *See e.g., COPE Case Report 00-20 on meeting proceeding. pdf, attached as Exhibit 3; COPE Case Report 00-28 redundant publication. pdf, attached as Exhibit 4; COPE Case Report 2004 redundant-publ.pdf, attached as Exhibit 5; COPE Case Report 2005 dublicate-publ. pdf, attached as Exhibit 6; COPE Report 01-11.pdf, attached as Exhibit 7; COPE Report 01-13.pdf, attached as Exhibit 8; COPE Report 01-18.pdf, attached as Exhibit 9; COPE Report 01-22.pdf, attached as Exhibit 10; COPE Report 01-25.pdf, attached as Exhibit 11; Duplicate publication: a joint statement from the Singapore Medical Journal and the Medical Journal of Malaysia, Peh W C G, Arokiasamy J T, Singapore Med J 2007; 48 (12), pages 1067-68* [stating, if editors inadvertently publish a duplicate publication, "[a] formal letter of

believe that these actions reflect the seriousness of the offence.")

Given The Final Report's three arguments in support of Dean Brueggemeier neglect to properly evaluate the facts in this case and "commonly accepted practices" within the scientific community, the Final Report must be revised. This revision must accurately evaluate Dean Brueggemeier's publications against the standards articulated in the aforementioned rules, procedures, and commentaries. This is because these rules, procedures, and commentaries properly detail the "commonly accepted practices that must be evaluated under Misconduct Policy §III(A)(2).

3. **The Committee Violated Misconduct Policy I(A)'s Obligation to " protect the integrity and reputation of research and scholarship produced by members of the University Community" By Finding It Could Not Address Violations of Journal Policies On Submission And Copyright.**

The Committee improperly claimed "violations of journal policies on submission and copyright are issues that need to be resolved between authors and journal editors." *Final Report p. 5.* This claim conflicts with commonly accepted practices within the scientific community. This conflict is evidenced in the following WAME statement:

> Journals do not have the resources or authority to conduct a formal judicial inquiry or arrive at a formal conclusion regarding misconduct. That process is the role of the individual's employer, university, granting agency, or regulatory body." http://www.wame.org/resources/publication-ethics-policies-for-medical-journals (19 of 26) 6/8/2008 12:13:36 PM

Moreover, many of the typical sanctions imposed by journals for publication policy violations contemplate investigations and sanctions issued by institutions like OSU. For instance, WAME identified the following sanctions - ranked in approximate order of severity – as sanctions journals impose for publication policy violations:

- A letter of explanation (and education) sent only to the person against whom the complaint is made, where there appears to be a genuine and innocent misunderstanding of principles or procedure.
- A letter of reprimand to the same party, warning of the consequences of future such instances, where the misunderstanding appears to be not entirely innocent.
- A formal letter referring the concerns to the relevant head of educational institution and/or funding body, with all the commentary and evidence collected by the journal. This will occur when it is believed that genuine misconduct is likely to have occurred, and its goal will be to submit the case for consideration of formal review and judgment by organizations better suited to that task than a peer review journal.
- A formal letter as above, including a written request to the supervising institution that an investigation be carried out and the findings of that inquiry reported in writing to the journal.
- Publication of a notice of redundant or duplicate publication or plagiarism, if appropriate (and unequivocally documented). Such publication will not require approval of authors, and should be reported to their institution.

- Formal withdrawal or retraction of the published in the journal, informing readers and the indexing authorities (National Library of Medicine, etc), there is a formal finding of misconduct by an institution. Such publication will not require approval of authors, should be reported to their institution, and should be readily visible and identifiable in the journal. It should also meet other requirements established by the International Committee of Journal Editors. *(www. icmje.org/#correct, accessed 12/2/03). http://www.wame.org/resources/publication-ethics-policies-for-medical-journals (23 of 26) 6/8/2008 12:13:36 PM (emphasis added)*

Therefore, the Committee should not issue a Final Report until it addresses whether Dean Brueggemeier's violations of journal policies on submission and copyright constitute misconduct under the Misconduct Policy.

4. **The Final Report Evidences Violations of Title VII's Prohibitions Against Retaliation By Highlighting An Inconsistent Application of the Misconduct Policy As Applied To Dean Brueggemeier and Dr. Sheryl Szeinbach.**

The Final Report evidences at least three examples of inconsistent application of the Misconduct Policy as applied to Dean Brueggemeier and Dr. Szeinbach. First, the Final Report refused to address any of Dean Brueggemeier's alleged "[v]iolations of journal policies on submission and publication . . . issues [because these issues] need to be resolved between authors and journal editors." *Final Report, at pg. 5.* But, OSU utilized Dr. Szeinbach's alleged violations of journal submission and publication policies as the basis of its investigation of Dr. Szeinbach's allegedly violated the Misconduct Policy. *See e.g., April 29, 2008 letter from OSU's Olga Esquivel-Gonzalez's to EEOC's Vicky Prior, pgs. 3-6. attached as Exhibit 12. See also, July 17, 2007 email Dr. from Jennifer S. Moseley to Dr. Sheryl Szeinbach, attached as Exhibit 13* (claiming a single instance of Dr. Szeinbach's republication of certain data violated commonly accepted scientific community practices because "the allegations here has preliminarily been found to have merit as determined by the resulting investigation that was conducted by the two journals . . . regarding similarities between the papers submitted.").

Second, the Committee ignored JCEM's finding that Dean Brueggemeier's duplicate publications and repeated use of the same figures without appropriate reference to the prior publication resulted in a censuring reprimand from the editor. *See, March 18, 2008 letter from Dr. Paul W. Ladenson to Dr. Brueggemeier.* Yet, OSU suggests Dr. Szeinbach engaged in misconduct by virture of her omission of a single reference – an action that both of the involved editors classified as an unintentional oversight. *Compare, November 6, 2007 Preliminary Report Of The Committee of Initial Inquiry Concerning Allegations of Misconduct In Research or Scholarly Activities, with August 15, 2007 email from Dr. Mark Levy to Dr. Szeinbach, attached as Exhibit 14.*

Third, in commenting on Dr. Szeinbach's use of a previously published database to address a different research question, OSU stated:

The essence of any published article that represents new scholarship is that

a new and unique scientific argument is presented to best describe and illuminate a new discovery. Self-plagiarism is sloth and defeats a presentation of such an argument. This practice diminishes the authors and damages the reputation of the institution.

Nevertheless, when JCEM determined Dean Brueggemeier engaged in redundant publication, the Committee defended his actions. The Committee's actions are extremely troubling because Dean Brueggemeier's publications appear to evidence a pattern of redundant publications spanning years. On the other hand, Dr. Szeinbach merely used a previously published database to address a different research question – an action far less egregious than Dean Brueggemeier's redundant publications.

This double standard is also extremely troubling because three of the four committee members charged with investigating Dean Brueggemeier also investigated Dr. Szeinbach. Therefore, unless this double standard is immediately corrected only one conclusion appears evident - OSU is allowing its Misconduct Policy to be applied in a discriminatory manner.

5. **The Committee Lacked a Valid Basis For Alleging Dr. Enrique Seoane-Vazquez's Complaint Against Dean Brueggemeier Constituted a "Frivolous Charge."**

In labeling Dr. Seoane-Vazquez's complaint against Dean Brueggemeier "frivolous," the Committee violated §III(A)(3) of the Misconduct Policy. This provision identifies "frivolous charges" as charges "unsupported by credible evidence . . . made in bad faith or with malice . . . ." In this case, overwhelming evidence proves Dr. Seoane-Vazquez's complaint is not frivolous. This evidence includes the rules, policies, and commentaries identified above. The evidence also rests in the findings of the editors of two journals who have independently concluded that Dr. Brueggemeier published unauthorized duplicate publications.

In accusing Dr. Seoane-Vazquez of engaging in "frivolous" conduct, the Committee violates Misconduct Policy §V(L) which prevents any OSU official from retaliating against a good faith complainant. And, it provides further evidence that OSU is retaliating against Dr. Seoane-Vazquez of his Title VII claims against OSU. As a result, all references to any alleged wrongdoing by Dr. Seoane-Vazquez should be removed from the Final Report.

6. **The Committee Ignored Evidence That Dean Brueggemeier Violated Misconduct Policy I(D)'s Confidentiality Provision.**

Although the Committee ventured outside its assigned task to inappropriately label Dr. Seoane-Vazquez's complaint frivolous, it ignored evidence of Dean Brueggemeier's clear violation Misconduct Policy I(D)'s confidentiality provisions. Dean Brueggemeier violated this provision by detailing OSU's confidential misconduct inquiry into Dr. Szeinbach to Cancer Letter's Editor Manfred Schwab. *See, May 11, 2008 Letter From*

Dean Brueggemeier To Manfred Schwab.

Instead of detailing Dean Brueggemeier's violation of the Misconduct Policy's confidentiality provision, however, the Committee issued a warning to Dr. Schwab. Specifically, the Committee stated, "Dr. Schwab should discontinue communicating the status of submitted manuscripts to outside individuals who are not directly concerned with manuscript submission. *Final Report, p. 5.* This warning was directed at Dr. Schwab's communications with Dr. Kostenbauder. But, these communications are perfectly understandable since Dr. Kostenbauder initiated Dr. Schwab's investigation into Dean Brueggemeier's duplicate publications in Cancer Letters.

Unfortunately, it appears OSU is selectively enforcing the confidentiality provisions of the Misconduct Policy. In addition to overlooking Dean Brueggemeier's violation of Misconduct Policy I(D)'s confidentiality provisions, OSU also overlooked Dr. Balkrishnan's violation of this policy. Dr. Balkrishnan violated this policy on April 28, 2007 when he detailed the confidential proceedings pending against Dr. Szeinbach to various OSU faculty members and individuals involved in the International Society For Pharmacoeconomics and Outcomes Research – an international organization to which Dr. Szeinbach serves.

Therefore, OSU should cease its selective enforcing the confidentiality provisions of the Misconduct Policy and reprimand both Dean Brueggemeier and Dr. Balkrishnan for violating that policy.

7. Concluding Thoughts

Given at the Final Report contains the abovementioned six violations of the Misconduct Policy, Dr. Seoane-Vazquez respectfully requests the Committee be disbanded and reorganized with new committee members. If this occurs, it is our hope that the new committee will secure the tools needed to properly evaluate Dean Brueggemeier's unauthorized duplicate publications under the Misconduct Policy.

If the Committee has any questions, or requires any additional documentation, please feel free to contact me. Please note, however, that in addition to my law practice, I am the executive director of Trade Justice Mission - a Christian NGO providing economic development opportunities to impoverished women in the developing world. My work with Trade Justice Mission will take me to Cambodia and Thailand from July 13, 2008 to August 4, 2008. Nevertheless, I will be happy to respond to the Committee either before I leave or after I return.

I thank you in advance for your assistance in finding a fair and equitable method for handling Dean Brueggemeier's duplicate publications under the Misconduct Policy.

Sincerely,

Eric Rosenberg

Enclosures
Cc: Dr. Enrique Seonane-Vazquez (via email).

SCIENTIFIC INTEGRITY MATTERS (REALLY MATTERS)

Dr. A. Douglas Kinghorn
Jack L. Beal Professor and Chair
Editor-in-Chief, *Journal of Natural Products*

College of Pharmacy, The Ohio State University, Columbus, OH

THE OHIO STATE UNIVERSITY

SEMINAR OUTLINE

- **Definitions (e.g., Scientific Integrity; Ethics; Plagiarism; "Self Plagiarism"; Fabrication; Falsification)**
- **Honest Errors Leading to Intentional Fraud**
- **Ethical Behavior and Scientific Publishing**
- **Consequences, Actions, and Resources**

DEFINITIONS

SCIENTIFIC INTEGRITY

- **Integrity is defined as (a) the firm adherence to a code of especially moral or artistic values. (b) The quality of being complete or undivided (*Merriam-Webster's Collegiate Dictionary*, 10th Ed., 1998).**

- **Among the assets most valuable to the researcher is a commitment to standards and professionalism that support good science.**

ETHICS AND ETHICAL PROFESSIONAL CONDUCT

- "Ethics" refer to a set of moral principles or values. Another way of expressing this term is "the discipline dealing with what is good and bad and with moral duty and obligation".

- "Ethical professional conduct" refers to conforming to accepted professional standards of conduct.

PLAGIARISM AND "SELF PLAGIARISM"

- "Plagiarism" is the intentional or unintentional use of another person's words or ideas.

- "Self-plagiarism" is a variant in which an investigator attempts to publish or submit the same information verbatim the in two or more manuscripts or other written material.

TYPES OF PLAGIARISM

- **Deliberate plagiarism is an attempt to claim credit for written work that was actually performed by someone else.**
- **Plagiarism may include failing to cite quotations and borrowed ideas.**
- **Accidental plagiarism may result from inappropriate citation and referencing.**
- **Plagiarism detection software includes "Turnitin" (http://turnitin.com/static/home.html).**

(http://en.wikipedia.org/wiki/Plagiarism)

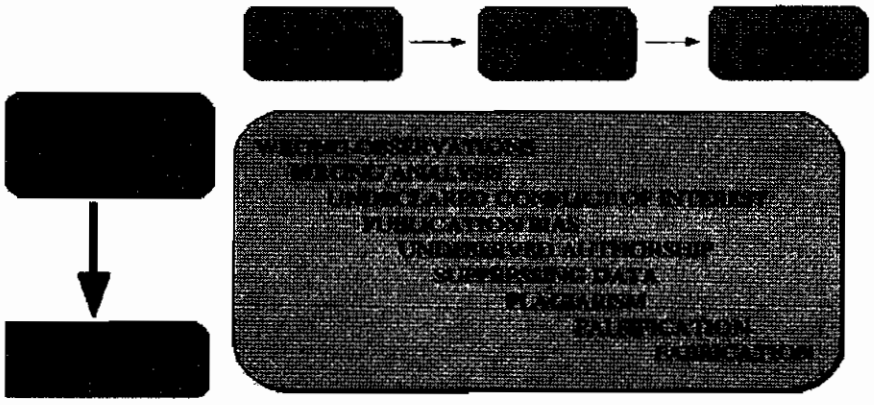
TWO ADDITIONAL EXAMPLES OF SCIENTIFIC MISCONDUCT

- **Falsification (altering truthful information).**
- **Fabrication (inventing information where none previously existed).**
- **The federal policy on research misconduct, formally defining these terms is at http://www.ostp.gov/html/001207_3.**
- **There is an Office of Research Integrity for laboratory personnel (including graduate students) at http://ori.hhs.gov).**

(See also Nylenna and Simonsen, *The Lancet* 2006, *367*, 1884 and Couzin and Unger, *Science* 2006, *312*, 38-43)

HONEST ERRORS LEADING TO INTENTIONAL FRAUD

PROGRESSION OF HONEST ERRORS TO INTENTIONAL FRAUD



(Adapted from Nylenha and Simonsen, *Lancet*, 2006, *367*, 1882-1884)

ETHICAL BEHAVIOR AND SCIENTIFIC PUBLISHING

PROBLEMS IN PUBLISHING!

- Reference will be made to recent experience received as Editor in Chief of the *Journal of Natural Products.*
- This is a co-publication of the American Chemical Society and the American Society of Pharmacognosy.
- Recently, ACS journals have seen more ethics violations than previously (Schulz, *C&E News*, 2006, April 10, pp. 62-65).

ACS JOURNALS

The Most Cited Journals in the Chemical and Related Sciences

- **Rank #1 in citations and/or ISI® Impact factor in all 7 ISI® core chemistry categories:**
  - **analytical, applied, inorganic & nuclear, medicinal, multidisciplinary, organic, and physical chemistry**
- **Rank #1 in citations and/or ISI® Impact factor in seven additional ISI® categories – from agriculture and environmental science to materials and polymer sciences, including the all-new ISI subject category of nanoscience and nanotechnology.**
- **Overall exceeded 1.13 million citations in 2005 AND 1 million article downloads PER WEEK, each and every week.**

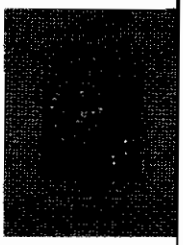
ACS JOURNALS

Biological & Medicinal Chemistry

- **12 ACS Journals in BIO/MED program.**
- **Nearly 100,000 peer-reviewed articles published in these titles alone.**
- **Long-established presence – and growing:**
  - ***Journal of Natural Products*** **(Volume 69)**
  - ***Journal of Medicinal Chemistry*** **(Volume 49)**
  - ***Biochemistry*** **(Volume 45)**
  - ***Biotechnology Progress*** **(Volume 22)**

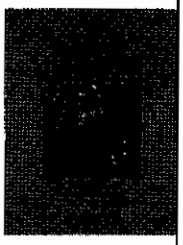
JOURNAL OF NATURAL PRODUCTS

- **Published monthly.**
- **2006, Volume 69, 12 Issues.**
- **"A Premier Arena for Natural Products Research".**
- **More than 8,800 peer-reviewed articles published since 1979.**
- **More than 4,000 articles in ACS Legacy Archives—all volumes published from 1979 to 1995 recently added.**



2005 ISI® JOURNAL CITATION REPORTS

- **Recorded nearly 10,000 citations in 2005.**
- **13% increase in citations in 2005 over 2004.**
- **Has nearly doubled number of citations in last 5 years.**
- **ISI® Impact Factor of 2.267 is highest in its history.**



PUBLISH WITH PRIDE!

- A well-crafted manuscript is an author's "window to the world", and serves as an advertisement as to the quality of the work being performed in his or her laboratory.
- Publication should always be done with pride, since a bad paper will be widely accessed electronically with minimal effort by the reader. This can haunt an investigator for years!

SOME ATTRIBUTES OF A WELL-PREPARED MANUSCRIPT

- Scientific manuscripts should be logical, factually accurate, concisely written, and afford adequate attribution to previous work on the same topic.
- Papers should conform to the technical scope of a selected journal, and be presented in the correct journal format.
- The rationale for the study being conducted should be explained.
- The submitted manuscript should be seen and approved by all co-authors.
- Internal "peer review" of the paper is highly desirable prior to formal submission.

WHO SHOULD BE INCLUDED AS A CO-AUTHOR?

- They should: (a) make a substantial and new contribution to the research; (b) take responsibility for some of the content of the manuscript; (c) read and agree to the manuscript before submission; and (d) agree to be named as a co-author.
- In practice, great reliance is placed on the integrity of the corresponding author to deal with the inclusion and ordering of co-author names.
- The corresponding (lead) author (designated with an asterisk) is usually head of a lab or a project, with a permanent or stable address (this is needed in case of queries about the paper).

GHOST AUTHORS VERSUS GUEST AUTHORS

- Two further definitions relating to misconduct in scientific publication are:
  "Ghost Authors" (individuals who should have been included in a scientific paper or presentation)
  "Guest Authors" (persons who do not fulfill authorship criteria)
- See Claxton, *Mutation Res.* 2005, *589*, 17-30 and 2005, *569*, 31-45 for two thoughtful reviews on scientific authorship. (Part 1. A window into scientific fraud? Part 2. History, recurring issues, practices, and guidelines).

COMMON PROBLEMS WITH SUBMITTED MANUSCRIPTS

- The work described is only of marginal significance (representing the "least publishable unit").
- Factual inaccuracies evident.
- Represents fragmentation of effort on same topic (e.g., the constituents of the same organism, in the case of natural product papers).
- Contains unnecessary components (e.g., unneeded compound trivial names or biological activity of threadbare significance).
- Poorly written (e.g., repetitive, meandering, expressed in "lab language"; lack of adhesion to required journal format).

OTHER SCIENTIFIC INTEGRITY PROLEMS IN SUBMITTED MANUSCRIPTS

- Submission of paper by inappropriate corresponding author, without appropriate permission (e.g., an ex-graduate student or postdoctoral).
- An institution where the work is performed (in part or in full) is not included in the list of addresses (as well as the reverse situation).
- Submission of same paper to two different journals.
- No permission obtained for exporting and importing organisms from the country of collection to the country where the laboratory work is conducted (specific to natural products papers).

PEER REVIEWING OF TECHNICAL MANUSCRIPTS

- Being asked to serve as peer reviewer by a journal editor is a significant professional function for a scientist.
- To participate in this manner, referees should normally have a terminal degree in the discipline concerned.
- Reviewers should have relevant expertise in the sub-discipline covered by the paper being reviewed.
- Reviewers must be willing to spend the necessary personal time to perform a thorough review.

PEER REVIEWING OF TECHNICAL MANUSCRIPTS

- Reviews should be impartial and offer constructive criticism.
- A thorough check for the novel aspects (e.g., whether a structure is really new) is needed.
- The correctness of the chemical and biological components of a paper should be checked.
- The literature review should be specifically examined for completeness and appropriate citation of previous literature.
- Suggestions for the improvement of rigor of the methodology used are very valuable.

CONSEQUENCES ACTIONS, AND RESOURCES

PLAGIARISM IN THESES

- To take a local example, a graduate student at Ohio University came forward in 2004, and pointed out that many theses in mechanical engineering at the university contained plagiarized passages.
- Altogether, 37 cases of plagiarism were found among graduate theses (*Science* 2006, *313*, 1041).
- The students concerned will have to defend their actions before a university panel (Research Integrity Committee).
- While some students may be allowed to rewrite their thesis or dissertation, degree revocation is possible, and notification of the problem in the student transcript may occur (*Columbus Dispatch* Sept. 6, 2006, p. A8).

ACTIONS THAT JOURNAL EDITORS MAY TAKE IN CASES OF ETHICS VIOLATIONS

- In cases of minor infractions, editors may simply explain problem(s) to corresponding authors.
- For blatant or repeated violations, the editor can forbid the author in question from submitting to the journal for a specified period.
- In the United States, in cases of disputed co-authorship, fraud, or plagiarism, the matter may be turned over to the institutional Office of Research Integrity of the institution of the lead author, in order to set up an inquiry.
- For overseas authors, the President of an institute or other organization to which the lead author belongs may be informed of the breach of ethics.

A TRUE CASE HISTORY

- A foreign postdoctoral student from an address in the U.S. submitted almost identical papers simultaneously to two different journals.
- This indiscretion was found by serendipity, and the journal editors contacted the director of the U.S. institution concerned to inform him/her of the problem.
- The postdoctoral tried to claim that the paper was rejected by one of the journals before being submitted to the second one.
- OUTCOME – The employment of the postdoctoral was terminated.

ETHICAL GUIDELINES FOR AUTHORS AND REVIEWERS

- The document "Ethical Guidelines to Publication of Chemical Research" is published annually by the American Chemical Society.

- A copy of the most updated version is being provided with today's seminar. Note in particular the ethical obligations of authors and reviewers.

A NEW ERA IN SCIENTIFIC INTEGRITY ACCOUNTABILITY

- Now that we are in middle of the first decade of the 21st century, research misconduct and lapses in academic and scientific integrity will not be tolerated.
- Ohio State graduate students should be aware of both the OSU Code of Student Conduct (http://studentaffairs.osu.edu/resource_csc.asp) and Ohio State's Ten Suggestions for preserving Academic Integrity (http://oaa.osu.edu/coam/ten-suggestions.html).

Wed, Mar 5, 2008 8:16 AM

From: Manfred Schwab <m.schwab@dkfz-heidelberg.de>
Reply-To: M.Schwab@dkfz-heidelberg.de
To: <drk@Columbus.rr.com>
Date: Wednesday, March 5, 2008 2:38 AM
Subject: publishing behavior

Dear Dr. Kostenbauder,

thanks so much for bringing to my attention the publishing behavior of Dr. Brueggemeier from Ohio State University. The Journal I am presenting is "Cancer Letters", this is with reference to publication number 4 according to your materials. This is a publication from 1999, at that time I have not been associated with the journal.

To start with, such behavior is totally unacceptable and, while it may be some sort of practice by some, it by no means is accepted by the scientific community - actually, it can be quite dangerous when brought to the attention of the relevant organisations (Faculty, Grant organisations).

Reality is that it does happen, probably more often than we would like to think. In the vast majority of cases it will not be identified. Given the fact that there are literally thousands of journals on the market, not even including open access online journals, that it almost impossible for the Editor or Reviewer to detect the duplicate publications. I admire your investigation skills, which have probably taken lots of time. However, I have hard time to think of a way for any Editor dealing with many manuscripts, or even reviewer, to do the same given the many other professional and scientific obligations that Editors and Reviewers have - none of these journal activities is a full-time job. Of course, I will add the name of this "scientist" to the list of "incriminated" persons, this list already does include some other names, and they will be banned from any publication in journals where I have some kind of influence. Other than this, I see little possibility to deal with this issue as an Editor.

Obviously, the most efficient way to deal with this would be if you send the materials to the Dean of the Faculty at Ohio State, or even to the grant support organisations. As I see from the publications, the author seems to have grant support, apparently even from NIH, which probably would be in great danger if such information would be transferred to them.

Again, I will monitor carefully any publication that might come in from this laboratory, and I again thank you for bringing this to my attention.

Exhibit 2

Kind regards,
Manfred Schwab
##########################
Manfred Schwab, Dr.rer.nat., Professor for Genetics
Director, Division of Tumour Genetics - B030
German Cancer Research Center (DKFZ)
Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany
Phone: 49-6221-423220; FAX---423281
GOOGLE search: Manfred Schwab Heidelberg
Lab website: http://www.dkfz.de/en/tumor-genetics/index.html
Editor:
Cancer Enzyclopedia: http://refworks.springer.com/cancer/
Cancer Letters: http://intl.elsevierhealth.com/journals/cale/

Case 00/20

Duplicate publication based on conference proceedings

A paper was submitted to Journal A and concern was raised by a reviewer that a substantial part of the paper had been previously published in two other journals. This point was taken up with the authors, who denied any lack of originality and maintained that their manuscript contained previously unpublished data. They did admit that part of the work had been presented as an invited lecture at an international conference and that three articles had subsequently been published, but had acknowledged this in their original submission letter. They had also included a copy of one of the published articles for information. The authors had also suggested that a member of the editorial board could act as the editor for their paper because he had been present at the conference and was aware of their presentation.

On inspection of one of the previously published articles in Journal B, it was found that it had been peer-reviewed, and that substantial parts had subsequently been reproduced in the paper submitted to Journal A: around 60 per cent of the introduction had been re-used. A forward by the editor of Journal B indicated that the papers had all been peer reviewed and based on a presentation at a conference. It was therefore not just another publication of conference "proceedings" and the copyright was owned by Journal B.

The editor of Journal A has no intention of publishing the article, but would like advice on what to do next.

Discussion/Advice

- The authors did disclose the other articles so they were partly in the right, and obviously not trying to be deceitful.
- It is also difficult to define as a percentage what constitutes duplication of previously published material, in which case it is difficult to take this any further.

Exhibit 3

Case 00/28

Plagiarism or redundant publication?

A manuscript was submitted with a covering letter clearly stating the originality and unpublished nature of the work. The authors stated that the results had already been orally presented at a meeting the previous year.

Before sending the manuscript for review the editors discovered that the manuscript contained 60% of the Materials and Methods text and 90% of the Results section of a previously published paper. Even the data were identical. Moreover, the authors' citation to the first article suggested that it was different from the current work (the corresponding author and the first author were the same in both manuscripts). The editors were not convinced that there was a deliberate attempt to mislead, but nevertheless they rejected the manuscript with a stern warning to both the first author and the corresponding author. As yet no reply has been received from any of the authors.

Discussion/Advice

- There had been some deception and a case of redundant publication, not plagiarism.
- The authors failed to declare an overlap, and citation is not enough.
- The authors should be given a chance to reply.
- A reminder should be sent (giving a time limited response), with reference made to the COPE guidelines otherwise the matter will be referred to the authors' institution.

Outcome

The authors emailed a detailed and apologetic reply. While the editors were convinced that there had been no deliberate attempt to mislead, the manuscript was rejected because a large part of the work had already been published.

Exhibit 4

Redundant publication (2004)

redundant publication; retraction

A complaint of redundant publication was made by a reader, who claimed that a second paper had been published in the journal, after the first had already been published elsewhere. No permission letter was obtained by the author of the second paper and the first paper had not been cited.

Action

- The editors should write to the authors and publish a retraction.
- The editors should write to the authors' institutions.

Update

- The editors decided not to write to the authors' institutions, but they have taken steps to write to the authors and publish a retraction.

Exhibit 5

Duplicate publication (2005)

duplicate publication; rejection of paper

A reviewer informed Journal A that a manuscript s/he had been asked to review was very similar to one s/he had reviewed for Journal B. The lead author was informed about this and told the editors would come back to him after discussing the matter further.

These discussions found striking similarities between the two papers and that the two manuscripts had been handled within the same time period. The Editorial Board concluded that the author had violated publication ethics for medical journals, and plans to reject the manuscript, explaining the reasons to the author, and informing Journal B of its findings.

Discussion

The steps proposed by Journal A were reasonable.

Action

None required.

Exhibit 6

Case 01/11

Duplicate publication

The editor of Journal A drew the attention of the editor of Journal B to two articles published in their journals which were remarkably similar. The editor of Journal A believed that certain passages of text suggested duplicate publication of results. The dates of publication indicated that these data were accepted first by Journal A. Should it turn out to be duplicate publication, the authors would have violated the requirements of every scientific journal for the submitted data to be original.

Both editors are aware that it's possible to unwittingly accept non-original, previously published data. But what can be done when that happens?

Discussion/Advice

- Both journals should publish a notice of duplication.
- Only the paper with the later acceptance date should be retracted.

Outcome

A notice of duplicate publication and withdrawal of the paper was published in Journal B. This was delayed while the university sought legal advice as to whether that intention might be considered defamatory. But the dean eventually phoned to say he approved of the proposed action.

The one author who was a qualified doctor, apologised, stating that as the two papers were aimed at different audiences, he thought publication would not be duplicated. He knows better now.

Case 01/12

Attempted redundant publication

A group of authors submitted a paper to Journal A, but the editor noticed that it was very similar to a paper already published in Journal B. Neither paper made any mention of the other in the text, references, or the covering letter.

The editor of Journal A sent a copy of the submission to the editor of Journal B who compared the two papers and decided there was substantial overlap. More worryingly, there seemed to be different numbers of eligible patients and different numbers randomised in the two papers, although they are supposed to be from the same study.

The editor of Journal B recommended the editor of Journal A to write to the authors asking for an explanation of the differences in patient numbers and their failure to reference the previously published paper.

What more should the editors of the two journals do?

Discussion/Advice

- The editor of Journal A should write to the authors asking for an explanation.
- A copy of this letter should also be sent to the editor of Journal B.
- If a satisfactory reply is not forthcoming then the editor of Journal A should refer the matter to the dean of the authors' institution, suggesting that an enquiry be started.

Outcome

The editor of Journal A wrote to the authors who have not replied.

Exhibit 7

Case 01/13

Duplicate publication

Sixteen randomly chosen papers were examined from a PubMed search of 370 publications between 1995–2000 by the same author.

Two papers were virtually identical, differing only in the form of the introductory paragraph and the list of authors. Neither publication acknowledges the other.

Another paper reported a "second ever published case", and two subsequent papers reported the same "second" case without reference to the earlier published paper. The text was again very similar.

Subsequently Journal A received a paper which was rejected. Apart from a change to the list of authors, it was identical to a paper that had been published two years earlier in a different journal. A paper with the same title and introduction had also been published in another journal. This could not be inspected as the journal is not available in any UK research library.

Two further manuscripts were submitted to Journal B, one in the form of a letter, and the second a full research paper. The letter was lifted directly from the paper; furthermore one of the tables was identical to that presented in the paper.

A further paper which had originally been rejected was resubmitted to Journal B, albeit slightly expanded, but with an entirely new list of authors. An independent statistician reviewed both papers and found that the content of two tables was identical except for the p values. Many of these had acquired a significance not suggested in the first manuscript.

Further to this example and the examination of just a few of the listed publications, clear cases of duplicate publication and attempted duplication were found. It's worrying that seemingly similar work can have different lists of authors, which suggests "gift" authorship. Changes in details of treatment and statistical significance throws the veracity of some of the work into question. Furthermore, the group's general failure to cite its own publications suggests a deliberate attempt to cover up duplication.

The editor of Journal B wants to inform the author that his publication will not consider any further submissions from this group. There is no guarantee that manuscripts would be original and issues of copyright are unclear. The editor would also like to alert the editors of the other journals involved.

Is this a reasonable course of action to take?

Discussion

- A wider enquiry would have to be made; merely writing to the authors would not be enough. Independent assessment had been obtained in which the author's misdemeanours were very evident.
- This was a matter for the author's institution(s) to investigate.
- Previous cases of gross duplicate publication had been detected by simply undertaking searches on MedLine.
- The role of all of the co-authors whose names appeared on several of the papers was also questionable, although many might be unaware of their involvement due to gift authorship. It would be unwise not to consider any publications from this group until all of the authors had been approached.
- The main issues for the editors were retraction and notice of duplication of known articles and referral of the authors to the head of their institutions, raising the issue of the wider crime of fraud.
- Overseas regulatory bodies often don't reply, perhaps because they are uninterested or feel it is not COPE's business to investigate misconduct.

Advice

- Check the submission letter to see if all of the authors have signed it.
- The editor should present a fuller version of the case presented at COPE to the corresponding author and all co-authors who were repeatedly linked to this work, asking for a response.
- If there is no reply, or only an unsatisfactory reply is received, then send a second letter asking for a response, giving them a set time limit in which to reply.
- If still no reply is received refer the matter to the authors' institution(s).

Exhibit 8

Case 01/13 (cont)

- The journal editors should jointly publish a retraction and unravel the story in an editorial.
- A further option would be to send a letter to a national journal such as *The Lancet* or the *BMJ*, exposing the duplication.

Outcome

The corresponding author had signed the submission letter on the other authors' behalf. In view of the large numbers of co-authors involved, the editor considered it impractical to write to them all, but contacted the editors of three other journals where there was evidence of duplicate publication.

One editor said that his journal was already refusing to consider any more work from the corresponding author. The other two editors indicated that they would take up the cases of duplication publication with the corresponding author. One of the three journals was in the process of publishing an apology, along with a fourth journal, concerning a separate case of duplication from this group.

The corresponding author had also been contacted and indicated that the cases of duplicate publication emanating from his group could have been due to insufficient care being exercised by some of his staff. After consulting the journal's editorial board the editor decided not to consider any further manuscripts from this group because they could not be confident that the work would be original.

Case 03/08

Is it duplicate publication when the first study is referenced in the second paper?

A paper entitled: "X and Y versus X alone for condition A in children" was submitted to Journal A and published in 2001. Journal A has since been alerted to a paper published in Journal B in 1999, entitled: "Comparison of combination of X and Y with X alone in the treatment of condition A," written by two of the four authors in conjunction with another author not listed on paper A.

Most of the abstract, methods, and discussion of the two papers are identical. The main difference is that paper B has four more patients in the study group and in Journal A all patients are referred as being 16 years old.

In Journal B, the authors mention that the treatment dose was lowered for children but do not identify how many of the study group were 16 years old. The figures in the two articles have identical axes but the curve is slightly different. Tabular data show the two papers' subject groups have different age ranges, but the breakdown of boys to girls is very similar as is the breakdown of the subtypes of the condition being treated.

All of the references in paper B are used in paper A, but the authors have added six extra references, one of which is the reference to paper B. The reference to paper B is made in the discussion section of paper A where the authors say: "Recently we reported that a combination of X and Y is a highly effective therapy for the treatment of condition A."

What should the editor do?

Discussion/Advice

- It was surprising that the editor and/or reviewer(s) didn't pick up on the fact that the reference was in the paper. But the onus is on the author to send in any papers that may have potential overlap with a submitted paper.
- This case was an example of poor behaviour on the authors' part.
- The second paper sounded like the same study, or perhaps a subset of the same study. It was not clear whether the author had made any form of declaration as to the earlier study other than the reference and brief mention of the first study in passing.
- Some journals now search and pull authors' references as a matter of course. This is primarily to find suitable reviewers, but often highlights duplicate papers.
- This is easier with an online submission system where a paper's references are automatically hyperlinked to the Medline reference.
- The difference of four patients suggests an element of deception.
- If he has not already done so, the editor needs to ask the authors to give their side of the story.
- The editor should check the initial submission letter to see if the author did make any kind of declaration about the other paper.
- The editor should also pursue this matter with the authors' employers and request an investigation.
- It is important to notify the author that the editor is planning on this course of action.
- The editor should contact the head of the authors' institution(s), as a department head would be too closely involved.
- Ultimately, the editor may have to withdraw the paper as its publication would skew data on the treatment being investigated.

Exhibit 9

Case 01/22

Yet more attempted duplicate publication

A study submitted to a journal was sent out for external review. The reviewer pointed out that it was essentially a shorter version of a paper already published elsewhere. The authors had referenced this paper, but did not make clear that the submitted paper was simply a summary of the other published paper. Nor did they mention the other paper in the covering letter, or include a copy of it. One of the differences between the two papers was that the original study had 17 authors, while the paper listed only 10.

The editor sent a stiff note to the authors saying that the journal regards this as poor publication practice. Should more be done?

Discussion

- Appropriate action had been taken by the editor.
- This is a further case for the record.

Case 01/23

Inadequately supervised research?

A piece of qualitative research was submitted that looked at the experiences of families facing a particular illness. The first author was both the main carer for the families and the researcher. She conducted and analysed all the interviews. Nobody else seemed to have analysed the verbatim transcripts, although two senior authors did help with analysis of the data.

The reviewers and editorial committee took the view that this research used wholly inadequate methodology and worried that the first author, who had undertaken the study as part of her PhD, had been inadequately supervised. The question was raised with all three of the authors.

The editor wrote to the supervisor, who it was suspected, would probably say that the methodology was acceptable and that s/he didn't agree with the objections raised.

Was this the right thing to do? Should more be done?

Discussion

- Many universities do not have trained supervisors nor do they provide guidance to supervisors as to their responsibilities.
- It is not only MSc and PhD students who are left unsupervised, and many lecturers are not trained to teach and often are appointed because they can attract large research grants, rather than for their teaching capabilities.
- This was qualitative research, much of which is very poor. For such a study to have any validity there must be two independent researchers.
- This case was also unusual in that the author was also the carer of the families. And the poor design of the study had not been picked up any independent scientific reviewers at the ethics committee stage.

Advice

- Await a response from the supervisor before taking any further action.

Outcome

Neither the lead author nor one of the supervisors accepted that there was any problem with the research.

The case was referred to the journal's ethics committee.

Exhibit 10

Case 01/25

Duplicate publication

An author published a paper in Journal A that looked extremely similar to one already published as guidelines in Journal B. Of 48 paragraphs of text, 41 were almost identical. It has since transpired that several authors who were involved in the writing of the article published in Journal B have not been acknowledged. Prior publication elsewhere had not been acknowledged in the Journal A paper.

The editor wrote to the authors requesting an explanation. He informed them that the journal takes a strong line on duplicate publication and disclosure of related publications, and that there should also be an appropriate acknowledgement of the contribution of other authors.

The editor also wrote to the editor of Journal A asking him to look at both of the papers and to give him his views.

Has enough been done?

Discussion

- This is a clear cut case of duplicate publication.

Advice

- Publish a notice of duplication in both journals.
- The editors of both journals should also write to the head of the authors' institution, informing them of this indiscretion.
- Inform the authors that this course of action is to be taken before writing to the institution.

Outcome

The author of the Journal A article contacted the editor of Journal B, stating that it was an error of omission and not a deliberate attempt to deceive. The editor accepted this explanation, but intends to contact head of the author's institution, and the authors have been informed of this.

No reply has been received from the editor of Journal A, but the editor of Journal B will attempt to find some agreed form of wording that both journals can publish.

Exhibit 11

THE OHIO STATE UNIVERSITY

Office of Human Resources

Organization and Human Resource Consulting
Suite 300
1590 North High Street
Columbus, OH 43201-2190
Phone: (614) 292-5688
Fax: (614) 292-0549

April 29, 2008

Ms. Vicky Prior
Investigator
Cleveland Field Office
AJCFB – Suite 3001
1240 E. 9th Street
Cleveland, OH 44199

RE: Charge of Discrimination
Sheryl L. Szeinbach 532-2007-02009

Dear Ms. Prior:

On November 21, 2007, I submitted a preliminary response to the charge of retaliation filed by Dr. Sheryl L. Szeinbach (hereinafter referred to as Charging Party), against The Ohio State University (hereinafter referred to as Respondent). At the time, Charging Party's claim of retaliation was premature as Respondent was still in the process of investigating the allegations brought forth against her. The process utilized by Respondent can be found at: http://orc.osu.edu/misconduct/index.cfm , a detailed and descriptive handbook at: http://orc.osu.edu/documents/keypamphlet.doc and the specific Research Misconduct policy at: http://orc.osu.edu/misconduct/policy.cfm

Since the investigatory process was still underway at that time, Respondent requested that the case be placed on "hold" until the Office of Research reached a decision regarding the allegation filed against Charging Party. At this time, the parties are still engaged in discussions in an attempt to resolve this matter. Consequently, Respondent's position is that no adverse action has taken place at this juncture since the matter is still pending.

The University's EEO Commitment.

The University maintains and is committed to a policy and practice of equal employment opportunity. See Exhibit 1. Specifically, this includes compliance with Title VII, ADA and Ohio's civil rights laws, and goes beyond that to embracing diversity.

Exhibit 12

In particular, The University takes allegations of discrimination very seriously and not only prohibits discrimination in the workplace, but also prohibits retaliation against any individual for filing a complaint. See Exhibit 2. Thus, Respondent's commitment to Title VII is unwavering.

Respondent Properly Managed the Outburst of one of the College's Faculty Members.

Charging Party noted in her particulars that on September 4, 2007, during a meeting of the graduate faculty from the Division of Pharmacy Practice and Administration, Dr. Rajesh Balkrishnan "clenched his fists and screamed at me threateningly, 'You just need to shut up and stop being a bitch.'" This allegation is accurate.

Upon notification, Respondent acted appropriately and in a timely manner to assess the situation and determine next steps. Both the Chair of the Department, Dr. Milap Nahata and the Dean of the College, Dr. Robert Brueggemeier engaged in a fact finding and made a determination that indeed Dr. Balkrishnan's behavior toward Charging Party had been inappropriate. On September 17, 2007, Dean Brueggemeier placed Dr. Balkrishnan on notice by stating that his behavior at the September 4th meeting "was very unprofessional, extremely rude and totally unacceptable. There is absolutely no justification for the use of the language nor the tone of your outburst at that meeting." See Exhibit 3. In this letter of counseling, Dr. Brueggemeier specifically instructed Dr. Balkrishnan "not to engage in discussions or interactions with Dr. Szeinbach, Dr. Seoane-Vazquez, or their students outside of classes." Moreover, Dean Brueggemeier sternly informed Dr. Balkrishnan that **his behavior would not be tolerated** and took several actions to address the behavior:

- He restricted Dr. Balkrishnan's access to development funds available through the Merrell Dow appointment;
- He told Dr. Balkrishnan that he was to engage a coach or a mentor; and
- He strongly recommended that he address the issue of anger management through the Faculty and Staff Assistance Program in the OSU Office of Human Resources. (A copy of the information about the services was attached to the memo). See Exhibit 4.

Therefore, Respondent acted within a reasonable period of time and with extreme care in managing Dr. Balkrishnan's behavior. Charging Party's claim lacks merit and must be dismissed.

Respondent Acted Appropriately in Addressing a Whistleblower Complaint Filed Against Charging Party.

In her complaint, Charging Party alleges that she was charged with "research misconduct by Dr. Brueggemeier (Dean of the College of Pharmacy) and Dr. Hayton (Dean of

Research)." This allegation is false. The allegation was NOT filed by Dr. Brueggemeier or Dr. Hayton.[1]

Rather, a whistleblower reported a complaint on Sunday, April 29, 2007 with then Provost Barbara Snyder[2] and with Dr. William Hayton. The reporter alleged that Charging Party engaged in research misconduct in connection with two (2) of her publications. See Exhibit 5. As is required under university policy, Provost Snyder forwarded the complaint to the Office of Research. The Office of Research is required to review and investigate allegations of research misconduct according to university policy. The Office of Research handles all allegations of research misconduct applying the same policies and procedures. Hence, upon receipt of this report, the Office of Research followed the policies, processes, rights and responsibilities delineated below:

- Policy: http://orc.osu.edu/misconduct/policy.cfm
- Overview: http://orc.osu.edu/misconduct/index.cfm
- Handbook: http://orc.osu.edu/documents/keypamphlet.doc

Initially, the allegation was reviewed by Dr. Robert McGrath, Senior Vice President for Research and Dr. Jennifer Moseley, Research Integrity Officer. The University's Interim Policy and Procedures Concerning Misconduct in Research or Scholarly Activities required that Dr. Moseley notify Dean Brueggemeier about the charges and of the need to conduct a preliminary review of the allegations. See Exhibit 6 at page 4. The purpose of this review was to determine whether sufficient evidence existed to warrant an inquiry and whether the charges fell within the definition of research misconduct. Dean Brueggemeier only reviewed the complaint because he was required to do so under university policy.

Upon review, BOTH Ms. Moseley and Dean Brueggemeier concluded that the charges contained sufficient evidence to warrant an inquiry, and that the charges fell within the definition of potential research misconduct. Dr. Moseley was not aware that Charging Party had engaged in any alleged protected activity at the time that she concluded that there was sufficient evidence to warrant a submission of the allegation to the Committee of Initial Inquiry.

On July 11, 2007, Charging Party requested that Ms. Moseley dismiss the allegations as frivolous. Ms. Moseley's response on July 17, 2007, clearly noted that:

> "This charge could not be considered frivolous under Section III.A.3 of the Research Misconduct policy. Rather, the allegation here has preliminarily been found to have merit as determined by the resulting investigation that was conducted by the two journals (Primary Care Respiratory Journal and Annals of

[1] The research misconduct allegation made against Dr. Szeinbach was made to Drs. Snyder and McGrath on Sunday April 29, 2007 – well before the remarks made by Dr. Balkrishnan in the September 4, 2007 graduate faculty meeting.

[2] Ms. Snyder is no longer an employee of The Ohio State University. She is currently President at Case Western Reserve University.

> Allergy Asthma & Immunology), in consultation with PubMed/Medline, regarding the similarities between the papers submitted. Specifically, the editors concluded that 'in our opinion, this was a significant oversight and does not reflect the standards of normal professional practice.' Further, they continued, 'we particularly draw your attention to the definition of redundant or duplicate publication.' Therefore, as determined through the Preliminary Review and again through the Alternative Resolution, the University concludes that it must continue to proceed with its Initial Inquiry into the allegations of research misconduct. Specifically, the charges include practices that seriously deviate from those that are commonly accepted within the relevant scholarly community based on the complainants' allegation and the resulting Journal editor's investigation." See Exhibit 7.

Based on Dr. Moseley's and Dean Brueggemeier's initial finding, university policy required that a Committee of Initial Inquiry be formed. The function of this body is to make a preliminary evaluation of the available evidence and testimony of the Respondent, Complainant and key witnesses and then determine if sufficient evidence of research misconduct exists to warrant an investigation under the University's disciplinary rules.

In August 2007, the Committee of Initial Inquiry was formed to review the allegations. The Committee members were:

- Dr. Charles L Brook (Chair), Professor, College of Veterinary Medicine
- Dr. Dale D. Vandre, Associate Professor, College of Medicine
- Dr. A. Douglas Kinghorn, Jack Beal Professor and Chair, College of Pharmacy

None of the Committee members were aware of Charging Party's alleged protected activity when they met and reviewed the complaint as well as other relevant materials. The Committee identified four issues and found, in part, as follows:

> **"Most of the prose in the 2007 article has been directly taken from the 2005 article; this is self plagiarism by the definition of many national funding agencies. The Committee agreed that the practice of using large sections of previous works, particularly without citation, represents the poorest of scholarly practices. The essence of any published article that represents new scholarship is that a new and nnique scientific argument is presented to best describe and illuminate a new discovery. Self plagiarism is sloth and defeats a presentation of such an argument. This practice diminishes the authors and damages the reputation of the institution."** (Emphasis added to the original)...
>
> **"Based on the definition of prohibited practices and the extensive experience of the committee in the publication of scientific findings, the Committee believes that the failure to quote the 2005 article in the 2007 article seriously deviates from commonly accepted practices within the research community and as such represents misconduct. The committee fails to understand that citation of the 2005 article would not occur as a required action to the senior author who not only had written the 2005 article but used its text and data extensively in**

preparation of the 2007 article. Based on the evidence presented to the committee, we find that an error is likely not an honest error in the conduct or interpretation of the research. Rather the committee sees the omission as a probable mechanism to hide the clear relationship between these articles." (Emphasis added to the original).

Based on the above findings, the Committee of Inquiry proposed to forward one finding of research misconduct. See Supra Exhibit 8. Subsequent to this determination, the Committee received a rebuttal from Charging Party dated November 20, 2007. See Exhibit 8 (a). The Committee convened on December 5, 2007 to reconsider the findings of its preliminary report. Committee members maintained their unanimous votes on three of the issues and a two (2) to one (1) vote concerning its earlier finding on Charging Party's failure to cite the article.

On January 9, 2008, the Committee made an official finding that sufficient evidence of misconduct existed to warrant an investigation under University Rule 3335-5-04. See full text at: http://trustees.osu.edu/rules5/ru5-04.php see also Exhibit 8 (b).

It is evident that in making this finding the Committee of Initial Inquiry placed due consideration to, among others factors, the Editorial statement issued by the Primary Care Respiratory Journal titled "Authors' responsibilities: guidance for submission of manuscripts to medical journals" and the accompanying "See Correction by Szeinbach on page 257." See Exhibits 9 and 10 respectively. The Editorial Statement stated in part as follows:

> "This Journal recently published a paper which, unknown to the editors – **there was no author declaration of any previous related publication** – presented an analysis of data previously used to answer a different research question in an earlier paper published in another journal, the *Annals of Allergy, Asthma and Immunology*. It is perfectly acceptable to use the same dataset in order to answer different research questions…**However, it is the responsibility of authors to ensure that previous publications, particularly those using the same dataset, are cross-referenced when reporting. This did not occur in this instance,** and resulted in a complaint to the editors…A full investigation ensued, involving the Editor-in-Chief, Deputy Editor, Assistant Editors and publishers of the *Primary Care Respiratory Journal* (PCRJ), and the Editor-in-Chief of the *Annals of Allergy, Asthma and Immunology (AAAI)*. (For investigation results see Exhibit 11).
>
> We have concluded that the paper submitted to the *PCRJ* was not a duplicate publication in terms of the study aims and outcomes. However, there is no doubt that substantial parts of the text of the *PCRJ* paper – including parts of the introduction, methods, results and discussion sections – are extremely similar to the paper published previously in the *AAAI*. In addition, the authors had not declared to the editors (and therefore to the referees) of the *PCRJ* the fact that the *AAAI* paper – in which they had used the same dataset – had been published. Furthermore, in not referencing the *AAAI* paper they did not permit readers of the *PCRJ* to put the later *PCRJ* paper in context. A correction is published in this issue of the *PCRJ*.

> ...We also wish in this editorial to draw the attention of future authors to the requirements relating to, and the ethics of, the submission of manuscripts for publication in medical journals.
>
> **According to the ICMJE guidelines, "when submitting a paper, the author must always make a full statement to the editor about all submissions and previous reports that may be regarded as redundant or duplicate publication of the same or very similar work. The author must alert the editor if the manuscript includes subjects about which the authors have published a previous report or have submitted a related report to another publication. Any such report must be referred to and referenced in the new paper. Copies of such material should be included with the submitted paper to help the editor decide how to handle the matter."**
>
> **It is the responsibility of authors to be aware of published guidance on the requirements for manuscripts submitted to medical journals, and to read carefully and understand the legal documents which they sign when submitting papers for publication."** See Supra Exhibit 9. (Emphasis added to the original).

In the referenced **"Correction"** noted above, Charging Party stated:

> "In our article entitled "*The impact of allergic rhinitis on work productivity*"...we were remiss in not acknowledging the use of the same data source, data collection and background literature that was used in our previous study addressing a different issue relating to lifestyle productivity which was published in the *Annals of Allergy, Asthma & Immunology (AAAI)* in 2005. We were also remiss in not referencing the previously published *AAAI* paper in the manuscript which we submitted to the *PCRJ*." See Supra Exhibit 10.

Subsequent to the above finding and in a good faith effort to resolve this matter as provided for in Section V.A. of the University's Interim Policy and Procedures Concerning Misconduct in Research or Scholarly Activities, Dr. Todd Guttman, Associate Vice President for Research Compliance and Interim Research Integrity Officer, issued a letter dated January 25, 2008, to Drs. Brueggemeier, Szeinbach, a third party and McGrath. See Exhibit 12. Dr. Guttman's goal was to explore and implement a resolution to this pending dispute. The parties met on March 7, 2008 to focus on an alternative resolution as provided in the policy instead of moving forward with an action under University Rule 3335-5-04.

On April 4, 2008, Charging Party issued a statement indicating that upon her review, the sanctions proposed by the parties during the March 7th meeting were unacceptable. See Exhibit 13. As such and in accordance with university rules and protocols Respondent will move forward with its process. See Exhibits 14 and 15.

All of the above steps reflect an extraordinary effort by Respondent to appropriately address and resolve this serious matter. Most critically, in carrying out its research mission, The Ohio State University expects members of the faculty to engage in research and to publish that research in a manner that protects both the integrity and the reputation

of research and scholarship produced by members of the University community. It is clear by the Committee's finding and the editors' highlight that the reputations of both Charging Party and the University were adversely impacted in this process.

Based on the reasons noted, Charging Party's allegation lacks merit and must be dismissed.

Respondent has Appropriately Addressed other Allegations of Research Misconduct.

Charging Party alleges that other individuals have engaged in research misconduct similar to the charges she is facing, but proceedings have not been initiated against them. This allegation is false. There was a prior formal complaint of research misconduct involving the College of Pharmacy, however, the records and the identity of Respondents and Complainants in such proceedings are to be kept confidential. Please see 42 CFR §93.108 at http://ori.dhhs.gov/documents/42_cfr_parts_50_and_93_2005.pdf, which in part states:

§ 93.108 Confidentiality

(a) Disclosure of the identity of respondents and complainants in research misconduct proceedings is limited, to the extent possible, to those who need to know, consistent with a thorough, competent, objective and fair research misconduct proceeding, and as allowed by law. Provided, however, that:

(1) The institution must disclose the identity of respondents and complainants to ORI pursuant to an ORI review of research misconduct proceedings under § 93.403.

(2) Under § 93.517(g), HHS administrative hearings must be open to the public.

(b) Except as may otherwise be prescribed by applicable law, confidentiality must be maintained for any records or evidence from which research subjects might be identified. Disclosure is limited to those who have a need to know to carry out a research misconduct proceeding.

Respondent must respect this confidentiality requirement but will attest that the Office of Research has followed established policies and procedures in the management of all allegations of research misconduct and has applied them in a consistent manner. Consequently, Charging Party's allegation is baseless and must be set aside.

Conclusion

Charging Party has not presented any evidence that would support her allegation of retaliation. Indeed, Dean Brueggemeier did not file a complaint of research misconduct against Charging Party as she alleges. The only reason that the Dean became involved in the complaint was because university policy mandated that he be involved in reviewing the complaint. Moreover, an independent committee of three individuals reviewed the

complaint and found sufficient evidence of research misconduct on the part of Charging Party to warrant an investigation under University rule 3335-5-04. Hence, any adverse action suffered by Charging Party is based on her own actions, and not based on purported retaliation by the University. Thus, Respondent respectfully requests that your office apply the appropriate standard of deference to administrative decision makers at the College and the University.

Based on the foregoing facts, Respondent, The Ohio State University, requests that the Commission dismiss the charge of retaliation filed by Dr. Sheryl Szeinbach.

If you have any questions regarding this matter, please feel free to contact me at (614) 292-5688

Sincerely,

Olga Esquivel-Gonzalez, JD
Employment Law & Compliance Manager
Organization and Human Resource Consulting
Office of Human Resources
The Ohio State University

X-Original-To: szeinbach@pharmacy.ohio-state.edu
Delivered-To: szeinbach@pharmacy.ohio-state.edu
Subject: RE: Alternative Resolution Conclusion
Date: Fri, 13 Jul 2007 18:58:23 -0400
X-MS-Has-Attach:
X-MS-TNEF-Correlator:
Thread-Topic: Alternative Resolution Conclusion
Thread-Index: AcfCICWZHVFUqgHCSZmaNt1G9M9OcgDDSqpB
From: "Moseley, Jennifer" <moseley.28@osu.edu>
To: "Szeinbach" <szeinbach@pharmacy.ohio-state.edu>

Sheryl,

As we discussed briefly today, I forwarded your request for an alternative resolution of the research misconduct allegations made against you to Dr. Robert McGrath, Senior Vice President for Research. Dr. McGrath concluded that your request was consistent with the process provided by the University's Interim Policy and Procedures Concerning Misconduct in Research or Scholarly Activities Policy and authorized me proceed in contacting the other involved parties, as required by the Policy.

I then forwarded your request to Dean Brueggemeier who, after careful consideration, declined your request. As I understand, the Dean felt that the conclusion made by the editors of the two journals (Primary Care Respiratory Journal and Annals of Allergy Asthma & Immunology), specifically that, "in our opinion, this was a significant oversight and does not reflect the standards of normal professional practice", does not alleviate the University of its responsibility to continue with an Initial Inquiry under the Policy.

I provided the Dean's decision to Dr. McGrath, who agreed to proceed with the Initial Inquiry - as required by the Policy.

Please contact me if you have any further questions.

Thank you,
Jennifer

Jennifer S. Moseley, Ph.D.
Conflict of Interest Administrator
Research Misconduct Administrator
The Ohio State University
Office of Research Compliance
1960 Kenny Road
Columbus, Ohio 43210

614-292-0057 (direct)
614-688-0366 (fax)
moseley.28@osu.edu

From: Szeinbach [mailto:szeinbach@pharmacy.ohio-state.edu]

Exhibit 13

X-Original-To: szeinbach@pharmacy.ohio-state.edu
Delivered-To: szeinbach@pharmacy.ohio-state.edu
Reply-To: <marklevy@animalswild.com>
From: "Mark Levy" <mark-levy@btconnect.com>
To: "'Szeinbach'" <szeinbach@pharmacy.ohio-state.edu>
Subject: RE: Editorial
Date: Wed, 15 Aug 2007 07:20:27 +0100
X-Mailer: Microsoft Office Outlook 12.0
Thread-Index: AcfevEGm3pTCHHCKTW2tWCImZY3q6wARwPPw

Dear Sheryl

I can only apologise for this oversight, I did mean to do so..
It is unfortunate that the letter below has been sent out. This letter misinterprets and overstates the contents of our carefully written editorial, which concluded that although there was an oversight, this was not intentional. We have also published your correction at www.thepcrj.org, which is self explanatory.

Please copy this note from me to those in your department if you wish.

DR Mark L Levy FRCGP
Editor-in-Chief PCRJ.

Dr Mark L Levy FRCGP
Senior Clinical Research Fellow: Allergy & Respiratory Research Group
Division of Community Health Sciences, University of Edinburgh
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- www.alamy.com/stock-photography-contrib-browse.asp?cid=%7b11AF87AC-A4A7-4F77-A072-82D0A0ABD7B8%7d&name=Mark+Levy

From: Szeinbach [mailto:szeinbach@pharmacy.ohio-state.edu]
Sent: 14 August 2007 22:59
To: Mark Levy
Subject: Editorial

Dear Dr. Levy:

Exhibit 14

EXHIBIT 16

Office of Research

Senior Vice President for Research
208 Bricker Hall
190 North Oval Mall
Columbus, OH 43210-1321

Phone (614) 292-1582
Fax (614) 292-6602

July 16, 2008

CONFIDENTIAL
COURIER DELIVERY

Dr. Enrique Seoane-Vazquez
Assistant Professor
College of Pharmacy
129B Parks Hall
500 W Twelfth Avenue
CAMPUS

Dear Dr. Seoane-Vazquez:

As noted in my June 25, 2008 response, I referred Mr. Eric Rosenberg's June 24, 2008 correspondence containing your appeal to the Committee of Initial Inquiry for reconsideration of its Final Report. I have attached a copy of the Committee's conclusion reached after its reconsideration –finding insufficient evidence of research misconduct by Dr. Brueggemeier to warrant an investigation under the University's disciplinary rules.

As per the University's Policy of Misconduct in Research or Scholarly Activities, your allegation of research misconduct against Dr. Brueggemeier has been dismissed and this decision is final. If you have questions concerning the University's process, please contact me or Dr. Jennifer S. Moseley, Research Integrity Officer, at jennifer.moseley@orc.osu.edu.

Sincerely,

Robert T McGrath

Robert T. McGrath

cc: R. Brueggemeier
Office of Research Compliance
Office of Legal Affairs

Enclosure

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July 15, 2008

Dear Dr. McGrath:

This letter is in response to your June 27, 2008 request that the Final Report of the Committee of Initial Inquiry for the complaint submitted by Dr. Enrique Seoane-Vazquez regarding actions of Dr. Robert Bruggemeier be reconsidered in light issues and arguments brought forward in a June 24, 2008 correspondence to you from Mr. Eric Rosenberg. Mr. Rosenberg states that he represents Dr. Seoane-Vasquez and has written to you at his request.

The Committee of Initial Inquiry has carefully read the correspondence from Mr. Rosenberg. After discussing the issues raised in this document, we believe that the conclusions reached in our Final Report were both fair and accurate. Therefore, we believe that our Final Report should stand, including our finding insufficient evidence of research misconduct by Dr. Brueggemeier to warrant an investigation under the University's disciplinary rules.

We wish to directly address only one of the six issues raised by Mr. Rosenberg. That of potential conflicts of interest he believes may exist with Drs. Vandre and Kinghorn. Mr. Rosenberg correctly identified Dr. Vandre as a co-author on a published manuscript with Dr. Brueggemeier in 2001 (Journal of Steroid Biochemistry and Molecular Biology 78,145-156, 2001). The standard of the National Institutes of Health regarding potential conflicts of interest with Professional Associates indicates that publications in the last three years may present a potential conflict of interest but publications prior to that time shall not be considered to present a conflict of interest (http://grants.nih.gov/grants/peer/COI_Information.pdf). The publication in question was published in August 2001, longer than six years ago, and is not considered to present a conflict of interest. Further, Drs. Vandre and Brueggemeier have not conducted collaborative research since the project resulting in the 2001 publication was completed. Based on this information the Committee of Initial Inquiry does not recognize that Dr. Vandre has a conflict of interest.

Mr. Rosenberg also states that Dr. Kinghorn is both a co-author with Dr. Brueggemeier and is a subordinate. Dr. Kinghorn is a faculty member of the College of Pharmacy where Dr.

Brueggemeier is the Dean. Therefore, Dr. Kinghorn may be considered a subordinate of Dr. Brueggemeier. In addition, Dr. Kinghorn was a co-author with Dr. Brueggemeier in a manuscript published in April 2006 (Journal of Natural Products 69, 700-703, 2006). Dr. Kinghorn functioned as a consultant during the investigation of Dr. Seoane-Vasquez's complaint against Dr. Brueggemeier. Dr. Kinghorn was asked to be a consultant due to his extensive experience as an editor in the field of pharmacy. At no time did Dr. Kinghorn participate in the investigation. He was available and did answer questions regarding publishing practices in the field of pharmacy. He was not allowed to direct the course of the investigation or to vote when the committee made decisions regarding the complaints of Dr. Seoane-Vazquez against Dr. Brueggemeier. Based on Dr. Kinghorn's defined and limited participation, the Committee of Initial Inquiry does not accept that he had a conflict of interest.

Sincerely,

Charles L. Brooks
Professor

EXHIBIT 17

Pediatr Nephrol (2004) 19:1379–1384
DOI 10.1007/s00467-004-1588-8

ORIGINAL ARTICLE

Renee F. Robinson · Donald L. Batisky · John R. Hayes · Milap C. Nahata · John D. Mahan

Body mass index in primary and secondary pediatric hypertension

Received: 24 February 2004 / Revised: 1 June 2004 / Accepted: 2 June 2004 / Published online: 21 October 2004

Abstract The objectives of this study were (1) to determine the relationship of body mass index (BMI) to primary or secondary hypertension in children and adolescents and (2) to assess BMI at the age of onset of hypertension in children and adolescents. Patient demographics, BMI, family history, presentation of disease, etiology of hypertension, medication, laboratory data, and findings from other procedures were recorded for all patients with hypertension followed in the Pediatric Nephrology Clinic at Children's Hospital, Columbus, Ohio, over a 4-year period. In total, 314 patients were studied: 218 with primary hypertension and 96 with secondary hypertension. Our patient population (166 males, 148 females) was diverse in age (13±6.3 years) and ethnicity (237 Caucasians, 54 African-Americans, 23 other). BMI was greater in patients with primary (27.5±9.2 kg/m^2) versus secondary (23.9±9.3 kg/m^2) hypertension (P=0.002). Children with primary hypertension with an increased BMI presented at an earlier age than children with secondary hypertension and an increased BMI. The age of onset (10.5±2.6 years) in primary hypertension was related to increased BMI (r=0.12, P=0.001); however, there was no relationship between BMI and age of onset of secondary hypertension (P=0.21). Children whose family members had essential hypertension had increased BMI compared with children without a family history of essential hypertension. Based on the logistic regression model constructed from our data, the likelihood of primary versus secondary hypertension was influenced by the presence of family history of hypertension independent of presence of obesity in the child. In conclusion, increased BMI is more common in children with primary than secondary hypertension; earlier onset of primary hypertension in the pediatric population was associated with increased BMI; the assessment of BMI is important in the evaluation of secondary as well as primary hypertension; the role of obesity in the development of secondary as well as primary hypertension in children merits further study.

Keywords Primary hypertension · Secondary hypertension · Body mass index

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Introduction

Hypertension remains a major risk factor for the development of serious disease including stroke, myocardial infarction, coronary heart disease, and nephropathy as well as decreased cognitive function [1, 2]. Essential or primary hypertension is the most frequent type of hypertension in children as well as adults. Primary hypertension has no single known cause; risk has been linked to higher than normal body weight, sodium intake, cholesterol level, and family history [3]. Secondary hypertension is caused by diseases of the kidney, lungs, central nervous system, endocrine and vascular systems. In the adult population, increased awareness and focus on hypertension has led to identification of modifiable factors (e.g., diet, physical activity, body weight, blood glucose) and non-modifiable factors (e.g., age, race, genetics, gender) related to the risk for development of hypertension [4, 5, 6, 7, 8, 9, 10, 11]. In children and adolescents, body size (e.g., height, weight), gender, and age are important determinants of blood pressure, with obesity being a significant contributor to this health burden and its financial costs [12, 13]. Body mass index (BMI) has been increasingly used for assessment of obesity and heart disease and for monitoring in adults. Until recently, there was no consensus for normal BMI values in children and adoleseents and the use of BMI as an indicator (potential marker for risk of having or potentially having pediatric hypertension in the future) has not been evaluated.

Increased recognition of pediatric hypertension in recent years has led to increased focus on the etiology, identification, prevention, and management of hypertension in children and adolescents [12, 14, 15, 16]. A number of factors associated with primary hypertension in adults have been shown to contribute to elevated blood pressure in pediatric patients [17]. As both obesity and hypertension are becoming more prevalent, affecting tens of millions of children, adolescents, and adults in the United States, the contributions of lifestyle and genetics continue to be debated [18]. It is possible that the increased incidence of obesity has led to more early recognition of children and adolescents with essential hypertension. The objectives of this study were (1) to determine the relationship between BMI and either primary or secondary hypertension in children and adolescents; (2) to assess the correlation between age of onset at diagnosis of hypertension and BMI in pediatric patients; (3) to determine the interaction of obesity and family history in primary and secondary hypertension in children and adolescents.

Materials and methods

A database composed of all 321 hypertensive children and adolescents followed by the Pediatric Nephrology Division at Children's Hospital, Columbus, Ohio, over a 4-year period, was analyzed to determine the relationships of BMI, age of onset, family history, and hypertension. The database was developed for physicians and nephrology staff to monitor patient status and medical care. Anthropometric variables, including height in centimeters, body weight, and demographic data including age, race, and gender of the child were collected. Patient weight (to the nearest 0.1 kg) and height (to the nearest cm) was obtained for clothed, barefoot children utilizing the same scale and recorded in the medical record by one of two nurse clinicians on each clinic visit over the entire 4-year period. Standard training protocols were implemented in the clinic setting to reduce error and variability between nurse clinicians. Family history, suspected etiology of hypertension, presentation of disease (e.g., headache, dizziness), age at diagnosis, medication doses, regimen duration, and date of initiation of drug therapy were also obtained. Guidelines from the "Update on the 1987 Task Force Report on High Blood Pressure in Children and Adolescents" were used to determine if the child or adolescent had hypertension. Both systolic and diastolic blood pressures were obtained twice by the nurse with the appropriate-size cuff from the right arm of the seated patient by Dynamap 8100 and confirmed by oscillometric measure when possible during routine nephrology clinic visits. The average of two visits was used to determine if the child or adolescent had hypertension (either systolic or diastolic blood pressure greater than the 95th percentile based on guideline standards) [12, 19]. Standard training protocols were implemented in the clinic setting to reduce error and variability between nurse clinicians. Diagnostic test results (e.g., renal ultrasonography and biopsy results) and laboratory parameters were obtained from the patient medical records. BMI was calculated from initial clinic visits as weight in kilograms divided by height in meters squared, and values were rounded to the nearest tenth. BMI has been used as an assessment tool in adults; recently a consensus was established by the Centers for Disease Control (CDC) for normal BMI values in children and adolescents. In this study, children were determined to be "overweight" based on the CDC definition for children and adolescents, BMI for age at or above the sex- and age-specific 95th percentile cut-off points based on 2000 CDC growth charts for children and adolescents.

The Mann-Whitney test was used to determine if a significant difference in BMI and subsequent classification of the child as overweight was noted between children with primary and secondary hypertension. Wilcoxon rank sum test was used to assess if there was a difference in age, age at diagnosis, and laboratory parameters between normal and overweight children. The chi-squared test was used to assess whether patient history of umbilical arterial catheter use or prematurity, symptoms on presentation, or parental history of disease was related to weight of the child in this sample. It was also used to assess whether family history of renal disease or essential hypertension was related to the underlying type of hypertension in the child or adolescent. Pearson correlation (r) and the coefficient of determination (r^2) were utilized to assess strength and direction of the relationship between two continuous variables (BMI and age) and to determine if the extent of variability in BMI was explained by age of the patient and age at the time of diagnosis. Pearson chi-squared was utilized to assess strength of the relationship between family history of renal disease and the underlying type of hypertension in the child or adolescent. We utilized stepwise multiple logistic regression to develop a model to determine if there was an association between the categorical response variable, type of hypertension, and the continuous explanatory variables, age at diagnosis and BMI, and the categorical variables, gender, and race. Stepwise multiple logistic regression was also used to determine if the risk associated with the individual explanatory variables for family history (i.e., essential hypertension, stroke, myocardial infarction, diabetes, polycystic kidney disease, hyperuricemia, and undifferentiated renal disease) were associated with type of hypertension in the child or adolescent. Hypertensive children were further divided into two subgroups (e.g., greater than 12 years of age, "teenager", and 12 years of age or less, "child") and multiple logistic regression analysis was used to determine if there was an association between the explanatory response variables and type of hypertension based on the age of the patient at time of diagnosis.

Results

Demographics

Of the 321 patients in the database, 7 records with incomplete height and body weight were subsequently omitted from the analysis. Of the 314 remaining patients, 218 with primary hypertension and 96 with secondary hypertension were evaluated. Our patient population (166 males, 148 females) was diverse in age (mean 13±6.3 years) and ethnicity (237 Caucasians, 54 African-Americans, 23 other). The distribution of children with primary (70%) and secondary (30%) hypertension among Caucasian children did not differ from those African-American children with primary (64.2%) and secondary (35.8%) hypertension (χ^2=2.125, P<0.713). There was a difference in positive family history of hypertension between primary (51.2%) and secondary hypertension (25.8%) (χ^2=16.4, P<0.001). The relationship between the categorical variables, family history of hypertension, and type of hypertension was significant with the chi-squared test for association (χ^2=50.6, $P\leq$0.001) (Table 1).

Impact of obesity in children and adolescents with hypertension

Children with a family history of essential hypertension were more likely to be overweight (49.6%) than children with no family history of hypertension (31.5%)

Table 1 Variables assessed in the logistic regression model for type of hypertension

Age of onset of hypertension*
Body mass index
Gender
Race
Family history of essential hypertension*
Family history of stroke
Family history of myocardial infarction
Family history of diabetes
Family history of kidney disease*
Family history of hyperuricemia

* Factors found to be significant

(P=0.002). The percentage of overweight children did not differ between Caucasian (53.8%) and African-American children (46.2%) (χ^2=3.231, P <0.520), but was significantly higher among those with primary (53.0%) versus secondary (38.7%) hypertension (χ^2=5.7, P<0.017). Overweight children with hypertension were also more likely to be diagnosed with primary hypertension (75.2%) than hypertensive children of normal weight (64.3%) (P=0.047). As a continuous variable, calculated BMI was greater in children with primary hypertension (27.5±9.2 kg/m^2) than in those with secondary hypertension (23.9±9.3 kg/m^2, P=0.002) and significantly related to the underlying type of hypertension (z=–2.437, P=0.015). Children whose family members had essential hypertension had increased BMI values compared with children without a family history of essential hypertension.

Triglyceride values were also significantly higher in the overweight children (159.3±110.36 mg/dl) compared with children with BMI below the 95th percentile of the CDC 2000 growth chart (88.1±36.8 mg/dl) (P=0.009). There was no difference in cholesterol levels between overweight and normal-weight children. Symptoms such as headache and edema were significantly more common in overweight children (22.7%, 5%) than normal weight children (10.5%, 0.7%) (P=0.006 and P=0.03, respectively). Prematurity, however, was not associated with being overweight (4.3%) and was more prevalent in children who were not overweight (15.4%) (P=0.002).

The age of children with hypertension who were classified as overweight (16±4.9 years) was significantly greater than that of the children with hypertension who had normal weight (11±6.5 years) ($P \leq 0.001$). The age at diagnosis of disease or time of diagnosis (10.5±2.6 years) was related to BMI in children with primary hypertension (r=0.12, P=0.001); however, no relationship was noted between BMI and age of diagnosis of secondary hypertension (P=0.21). The age at time of diagnosis of hypertension in overweight children (12.7±4.7 years) did, however, differ from that of children of normal weight (8.5±6.1 years) ($P \leq 0.001$).

Interaction of obesity and other variables in relation to hypertension

Based on a scatterplot, we noted children with BMI over 80 kg/m^2 were in a different subset (n=8, primary hypertension=3, secondary hypertension=5) and were subsequently eliminated for the purpose of developing a regression model. Our results were used to construct a model to quantify the risk associated with the individual explanatory variables (e.g., age of diagnosis of disease, BMI, race, gender, and family history) in the development of either primary or secondary hypertension in our population. Of all variables examined, age of diagnosis of hypertension (AOH, P=0.001), family history of essential hypertension (HxHTN, P=0.002), and family history of renal disease (HxRD, $P \leq 0.001$) were the factors found to account for the most variability in type of hypertension (i.e., primary hypertension, secondary hypertension). Although each of the individual family history variables was significant, the interaction of family history of essential hypertension and obesity was not significant (P=0.089). There was no additive effect of obesity and family history of essential hypertension on the likelihood of the child having primary versus secondary hypertension. The likelihood of primary hypertension increased with older age of diagnosis of hypertension and increased with positive family history of essential hypertension. The likelihood of secondary hypertension decreased with older age of diagnosis of hypertension and positive family history of essential hypertension, but increased with positive family history of renal disease [y=–0.136–0.09(AOH)–1.00(HxHTN)+1.98(HxRD)].

Age of onset and type of hypertension

A separate logistic regression was used to determine the impact of age of diagnosis of hypertension. The population was divided based on age at time of diagnosis of hypertension (≤12 years of age and >12 years of age) and BMI, presence of obesity, race, and gender were evaluated. Family history of essential hypertension (HxFEH, P=0.08) was not significant in children ≤12 years of age at diagnosis with secondary hypertension, but was significant in children >12 years of age at diagnosis (HxFEH, P=0.03). Family history of renal disease (HxRD) was significant in both children ≤12 years of age and >12 years of age at diagnosis of hypertension (P=0.001 and $P \leq 0.001$, respectively). Obesity was a significant negative predictor in children ≤12 years of age at diagnosis with secondary hypertension (P=0.03), but was not predictive in children >12 years of age at diagnosis of hypertension (P=0.32). BMI was also a negative predictor of secondary hypertension in children ≤12 years of age at diagnosis of hypertension (P=0.01) but was predictive of primary hypertension risk in children >12 years of age at diagnosis of hypertension.

Discussion

Our study population was relatively diverse, consisting of children and adolescents of a variety of racial and ethnic backgrounds and ages across much of the state of Ohio; however, due to the small number of patients in other ethnic groups we are not able to generalize to populations other than Caucasian and African-American children and adolescents. However, the percentage of patients with primary and secondary hypertension was comparable between Caucasian and African-American children and was large enough to assess the risk factors associated with hypertension (family history, age of onset of disease, BMI, race, and gender).

Family history of hypertension has been associated with increased risk of hypertension and is considered to be a significant predictor of hypertension in children [15, 20]. There is strong evidence for the genetic basis for both essential and secondary hypertension and the genetic link between obesity and hypertension in adults [21, 22, 23, 24]. Heritability is believed to be between 30% and 50% for essential hypertension and up to 70% in hypertensive siblings [24, 25]. In our study, family history of hypertension was present in 51% of children with primary hypertension and in 26% of children with secondary hypertension. Family history of primary hypertension was more common in overweight children with hypertension than in normal-weight children with hypertension (P=0.002). The results of the multiple logistic regressions confirmed the association and quantified the risk associated with family history of essential hypertension, family history of renal disease, presence of obesity, and hypertension in the child or adolescent.

In children, blood pressure values are significant predictors of future blood pressure rank and appear to be maintained throughout adolescence and early adulthood [26]. Although the immediate end-organ risks of childhood hypertension appear to be small, evidence of cardiovascular and hemodynamic changes related to sustained hypertension has been documented in early adulthood [27, 28, 29]. Children with serial and isolated elevated blood pressure values (≥95th percentile based on height, weight, and age) are more likely to develop high blood pressure as adolescents and adults [12]. The onset of puberty, sexual maturation, and subsequent change in height and weight appear to influence the extent and rapidity of increase in blood pressure [14, 30].

The prevalence of childhood obesity has increased in recent years. A 10-year cohort study in the United Kingdom demonstrated a significant increase in overweight children from 1989 to 1998, especially in children less than 4 years of age [31]. Studies have shown that obesity, resulting from an imbalance between energy intake and expenditure, is likely to persist into adulthood, and that obesity increases the risk of specific diseases (e.g., diabetes mellitus, cardiovascular disease) and overall morbidity and mortality in adults [31, 32, 33]. In the 1970s, a nationwide screening program of more than 1 million adults confirmed the results of longitudinal and cross-sectional studies examining the relationship of body weight and blood pressure [34]. The presence of hypertension in overweight adults aged 20–64 years was greater than that of normal and underweight individuals [34]. This correlation between blood pressure and body size was supported by additional longitudinal and cohort studies in adults and children [14, 34, 35, 36, 37]. Controlling for the effects of family history, socioeconomic status, race, and gender, the prevalence of subsequent hypertension in young adulthood is higher in obese children than children with normal body weight [26].

The most recent National Health and Nutrition Examination Surveys (NHANES) indicated that 22% of American children were overweight, with an especially increased prevalence among African-American females and Mexican-American males [33]. Of note, BMI appears to be a significant predictor of blood pressure in both children and adolescents reflecting both body weight and height [26, 38]. The frequency of hypertension in obese individuals is twice that of self-reported normal-weight individuals across all genders, age, and race [34]. Studies in the United Kingdom [31] and the United States [39, 40] have demonstrated an increase in body weight and BMI in children over the past 10 years. The increase in incidence of body weight was noted irrespective of increases in height and was evident in children as young as 4 years of age [31]. Earlier studies have applied the standard adult value BMI greater than 25 kg/m^2 [41, 42]. The new standard for pediatric studies utilizes the CDC age- and sex-specific 95th percentiles and was used in our study.

The National Heart, Lung and Blood Institute Growth and Health Study, a 10-year multicenter study to investigate the factors associated with obesity in African-American and Caucasian adolescents, demonstrated racial disparities as early as 10 years of age, with more adiposity in African-American females at 12 years of age [43]. Factors such as energy intake and expenditure, earlier onset of menarche, genetic, and/or metabolic differences resulting in increased sodium retention and social factors such as diet, exercise, and energy expenditure may influence weight [43, 44, 45, 46]. In adults, educational level and age appear to be important factors in the likelihood of obesity; as the educational level of Caucasian and African-American adult females rises, weight decreases while education does not appear to be related to obesity in males. A strong correlation of obesity, measured by subscapular skinfold thickness, and blood pressure has been noted in Caucasian boys, Caucasian girls, African-American boys, and African-American girls, respectively (r=0.88, r=0.80, r=0.78, r=0.70) [37]. The effect of education and school performance on weight gain and hypertension still needs to be addressed in the pediatric population [47].

Investigators have found that both intermittent and ambulatory blood pressure measurements correlate with height, body weight, waist-to-hip ratio, and subscapular skinfold thickness in children and adolescents [41]. Short-term studies of blood pressure and the effect of aerobic fitness and BMI have demonstrated beneficial effects of

aerobic activity on blood pressure in children as young as 5 years of age [48]. In the landmark Framingham study of adults, the presence of and degree of obesity were related to the presence of hypertension [35]. In that study, race, gender, obesity, and BMI were not predictive of the underlying type of hypertension. In contrast, in our pediatric population, the presence of obesity did impact the type of hypertension and was independent of family history of essential hypertension or renal disease. However, it is important to note that our study was smaller and consisted of a homogeneous population (patients diagnosed with hypertension) compared with the broad-based Framingham study.

An independent risk factor for the diagnosis of primary hypertension in our population appears to be age at time of diagnosis of hypertension. The significance of age in blood pressure evaluation has been stressed in the past two Task Force reports on blood pressure in children [12, 19]. In our study, age of onset of disease was predictive of the underlying type of hypertension. Since increased BMI is associated with earlier onset of hypertension only in the group with primary hypertension, it appears that obesity may augment the development of this inborn trait.

Early identification of risk factors, prompt diagnosis, and effective treatment in the pediatric community may decrease the complications seen in adults with hypertension. In our population of children there are strong relationships between BMI, classification as overweight, family history, and underlying type of hypertension.

In conclusion, increased BMI is more prevalent in children with primary than secondary hypertension, and is more predictive of the underlying type of hypertension in children $\leq$12 years of age. BMI is associated with earlier age at diagnosis of primary hypertension and does not appear to be dependent on race or gender; however, overweight children with a family history of hypertension appear to be at increased risk for both primary and secondary hypertension. Age at diagnosis, family history of essential hypertension, and family history of renal disease are predictive of risk of developing hypertension and deserve to be considered in patient risk assessment of children with hypertension.

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AJH 2005; 18:917–921

Genetics

Significance of Heritability in Primary and Secondary Pediatric Hypertension

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Background: Patient weight and family history are significant risk factors for the development of hypertension in children. Multiple genetic factors have been identified in primary (essential) hypertension in adults; however, the delineation of genetic factors in the separate populations of children with primary or secondary hypertension are not well understood. Heritability is the proportion of observed variation in a particular trait that can be attributed to an inherited genetic factor in contrast to environmental factors. In the consideration of hypertension, heritability can be assessed in terms of an underlying continuous gradient of the liability for developing hypertension. With this assumption it is possible to compute heritability using hypertension incidence among relatives and described by Falconer. Heritability values range from 0 (no genetic contribution) to 1 (complete genetic contribution). The aim of this study was to determine the genetic contribution to primary and secondary hypertension in a pediatric population through heritability analysis.

Methods: This was a retrospective case-control analysis of medical records of children (n = 276) followed in the Pediatric Nephrology Clinic over a 4-year period from 1999 to 2002. There were 192 children and adolescents with primary hypertension (124 male, 68 female, age 0 to 21 years) and 84 children and adolescents with secondary hypertension (46 male, 38 female, age 0 to 21 years). Each hypertensive group served as the control for the other. Estimates of heritability were made using Falconer's method 2.[11] The model assumes independence between the environment and genetic factors and that the joint distribution of liabilities between parent and child are normally distributed. Problems can arise from computing heritability due to dominance within loci, correlations between nongenetic familial effects, or the presence of a major gene.

Results: Of the children and adolescents with primary hypertension, 49% had parents with primary hypertension; and of the children and adolescents with secondary hypertension, 24% had parents with primary hypertension. Of the children and adolescents with primary hypertension, 10% had parents with secondary hypertension; and of the children and adolescents with secondary hypertension, 46% had parents with secondary hypertension. The estimated heritability for primary hypertension was 0.84 (SE = 0.21). The estimated heritability for secondary hypertension was 1.14 (SE = 0.21). As the value was >1, this indicates that the fit of the liability model is poor and that a few genes, or even one major gene, were significantly involved in the causes of secondary hypertension in the children and adolescents studied.

Conclusions: The results suggest that primary and secondary hypertension do not share the same type of genetic profile. Primary hypertension in children and adolescents is likely due to a large number of additive contributions of genes, although a highly correlated environmental component can not be excluded. The continuous liability model is inappropriate for secondary hypertension because the estimate was substantially greater than one. This study supports the model that secondary hypertension in children and adolescents may be related to just a few genes. Am J Hypertens 2005;18:917–921 © 2005 American Journal of Hypertension, Ltd.

Key Words: Pediatrics, hypertension, heritability.

Hypertension remains a major risk factor for the development of serious disease including stroke, myocardial infarction, coronary heart disease, and nephropathy.[1,2] Essential or primary hypertension is the most frequent type of hypertension. It has no single known cause; however, risk has been linked to higher than normal body weight and family history of hypertension. Secondary hypertension is linked to diseases of the kidney,

Received August 3, 2004. First decision November 29, 2004. Accepted January 4, 2005.

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0895-7061/05/$30.00
doi:10.1016/j.amjhyper.2005.01.010

lungs, central nervous system, endocrine system, and vascular system. Increased awareness and focus on hypertension has led to identification of modifiable factors (such as diet, physical activity, body weight, blood glucose) and nonmodifiable variables (such as age, ethnicity, genetics, and gender) in the adult population.[3–6] In children and adolescents, body size (height and weight), gender, and age are important determinants of blood pressure (BP).[7] Body mass index (BMI) has been used as an assessment tool for obesity and more recently as a monitoring parameter for hypertension in adults; however, until recently a consensus was not established for normal BMI values in children and adolescents, and the use of BMI as an indicator in pediatric hypertension was not evaluated.[8]

Increased recognition of pediatric hypertension in recent years has led to renewed focus on the etiology, identification, prevention, and management of hypertension in children and adolescents.[7,9,10] A number of factors associated with primary hypertension in adults have been shown to contribute to elevated BP in pediatric patients. Patient weight and family history are significant risk factors for the development of hypertension in children. Multiple genetic factors have been identified in primary hypertension in adults; however, the delineation of genetic factors in the separate populations of primary and secondary hypertension in children and adolescents are not well understood.

Heritability is the proportion of observed variation in a particular trait that can be attributed to genetic factors in contrast to environmental factors. Heritability is estimated from the correlation among relatives. The degree of heritability in the etiology of a disease assumes an underlying continuous gradient of liability, which is a result of the accumulation of the additive contribution of many genes. The concept has been expanded by Falconer[11] to suggest a latent cutoff point on the gradient of liability above which the disease manifests itself sufficiently to be diagnosed and below which it is not. With this assumption it is possible to compute the association, heritability, using hypertension incidence among relatives. The objective of this study was to determine the genetic contribution to primary and secondary hypertension in a pediatric population through a heritability analysis.

Methods

A retrospective, case-control analysis of medical records of children and adolescents with primary and secondary hypertension (n = 276) followed in Pediatric Nephrology Clinic over a 4-year period from 1999 to 2002 was conducted. Demographic data, including height, body weight, age, ethnicity, and gender of the child were collected. Family history of hypertension or renal disease, suspected etiology of hypertension, presentation of disease (eg, headache, dizziness), date of diagnosis, medication doses, regimen duration, and date of initiation of drug therapy were also obtained. Each hypertensive group served as the control for the other group.

The inheritance of liability to diseases

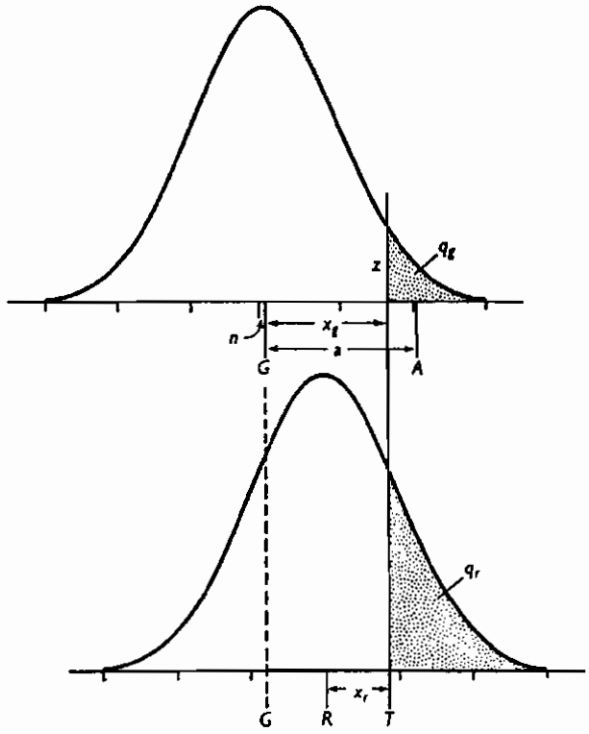

FIG. 1. Two distributions representing the general population (**above**) and the relatives of affected individuals (**below**) compared with reference to the fixed threshold (T). A = mean liability of affected individuals in the general population; a = mean deviation of affected individuals from the population mean (= z/q); G = mean liability of general population; subscript g = general population; n = mean deviation of normal individuals from the population mean (= z/[1 − q]), q = incidence, ie, proportion of individuals with liabilities exceeding the threshold; R = mean liability of relatives; subscript r = relatives; x = deviation of threshold from mean, ie, the normal deviate (note: usually annotated with z as in z-score or standard score); z = height of the ordinate at the threshold.

For a qualitative trait, heritability is defined as the proportion of variation attributable to genetic factors. Heritability (h^2) equals 2 times the slope of relatives' trait of interest regressed on the target patient (propositi) trait of interest when the relatives are full siblings, parents, or children. When the trait is discrete as in the case of the diagnosis of primary or secondary hypertension, then an assumption is made that there is an underlying continuous distribution of the risk (liability) of developing hypertension. Falconer has proposed a method of estimating heritability with incidence data.[11] Figure 1, based on the work of Falconer, illustrates the relationship between the liability distributions of the general population and the relatives of the affected population. The regression, b, is defined as:

$$b = (R - G)/(A - G) = (x_g - x_r)/a$$

G is the mean liability of the general population, A is the mean liability of affected individuals within the general

Table 1. Heritability of primary and secondary hypertension from the family history of the affected patients

	Primary Hypertension Heritability $h^2 = 0.80$ (SE = 0.19)			Secondary Hypertension Heritability $h^2 = 1.36$ (SE = 0.17)		
Family History of Hypertension	Secondary Hypertension Relatives	Primary Hypertension Relatives	Total	Primary Hypertension Relatives	Secondary Hypertension Relatives	Total
No	64	98	162	173	45	218
Yes	20	94	114	19	39	58
Total	84	192	276	192	84	276

Note: Relatives of the secondary hypertension patients serve as representatives of the general population when calculating heritability of primary hypertension. Relatives of the primary hypertension patients serve as representatives of the general population when calculating heritability of secondary hypertension.

population, and R is the mean liability of the relatives of the affected patients. The difference between the mean of the general population (G) and the mean of the relatives of the affected population (R) is the impact of the genetic relationship. The difference between the mean liability of the general population (G) and the mean liability of those individuals within the general population that are affected (A) provides a scale for the magnitude of the impact. The ratio of the two differences is the regression (b) of the relative's degree of liability on the degree of liability of an individual from the general population. Heritability (h^2) is 2 times the slope (b) when the relatives are full siblings, parents, or children. The complete calculation is described in the Appendix.

In this retrospective study, relatives of secondary hypertension patients served as general population controls to the relatives of patients affected with primary hypertension, and the relatives of primary hypertension patients served as the general population for the relatives of patients affected with secondary hypertension. This approach was taken because it was assumed primary and secondary hypertension are independent, the patients are served by the same clinic and thus the demographics are similar and the family history forms are identical. Each patient contributed one observation to the analysis in which any parent or full sibling either was or was not affected.

Results

The medical records of 192 children and adolescents with primary hypertension (124 male, 68 female, age 0 to 21 years) and 84 children and adolescents with secondary hypertension (46 male, 38 female, age 0 to 21 years) were reviewed.

Heritability of Primary Hypertension

Of the patients with primary hypertension, 49% had relatives with primary hypertension compared to 24% of the control patients with primary hypertension.

As shown in Fig. 1, the value of x_g is 0.71. Thus, it is assumed that when individuals in the general population with an underlying primary hypertension liability scale value that is >0.71 standard units, they will be diagnosed with primary hypertension. The incidence of primary hypertension among relatives of children and adolescents with primary hypertension was 49%, so the threshold cut at the standard score of 0.025 point on the normal distribution. The estimated heritability for primary hypertension was 0.80 (SE = 0.19). The heritability index suggests that 80% of the variance of liability of primary hypertension is attributed to additive genetic factors. Heritability of primary and secondary hypertension, based on family histories of the affected patients, is shown in Table 1.

Heritability of Secondary Hypertension

Of the patients with secondary hypertension, 46% had at least one relative with secondary hypertension, and of the patients with primary hypertension 10% had at least one relative with secondary hypertension. The estimated heritability for secondary hypertension was 1.36 (SE = 0.17). The computed value >1 suggests that an underlying liability scale of a continuous variable is not appropriate for these data. The liability model assumes the additive effects of a large number of genes. The failure of this assumption suggests that possibly a few genes or even one major gene are significantly involved in the causes of secondary hypertension in pediatric populations.

Discussion

In children, BP values are significant predictors of future BP rank and appear to be maintained throughout adolescence and early adulthood.[12] The tracking of BP measurements does not appear to differ between obese and normal-weight adolescents; however, the onset of puberty, sexual maturation, and subsequent change in height and weight appear to influence the extent and rapidity of increase in BP.[9] Although the immediate risk associated with hypertension in childhood is small, evidence of cardiovascular and hemodynamic changes consistent with sustained hypertension have been documented in early adulthood.[13–15] Children and adolescents with serial and isolated elevated BP values (≥95th percentile based on height, weight, and age) are more likely to develop high BP as adolescents and adults.[7] Therefore, early identi-

fication of populations at increased risk may be important to allow methods for early intervention designed to decrease end-organ damage in children and young adults.

There is strong evidence for the genetic basis for both essential and secondary hypertension and the genetic link between obesity and hypertension in adults.[16–19] Heritability is believed to be between 30% and 50% for essential hypertension and up to 70% in hypertensive siblings.[19,20] In our study we found that heritability was 80% for primary hypertension. Of the children and adolescents with primary hypertension, 49% had at least one parent with primary hypertension.

A similar number of parents of secondary hypertensive children and adolescents (46%) had secondary hypertension. The estimate of heritability of the children and adolescents with secondary hypertension was >1. Heritability represents a proportion of variance and cannot be >1, and thus this result indicates problems with applying the heritability analysis to this population. Problems in estimating heritability may be due to dominance within a loci and to correlations between the environment and genetic factors. The genetic component and environmental components are assumed to be independent. Failure to meet this assumption could also result in an overestimation of the genetic component.

In conclusion, the results suggest that the genetic contribution to hypertension is different for primary and secondary hypertension. Primary hypertension may be due to a large number of additive contributions of genes, although a highly correlated environmental component cannot be excluded. The continuous liability model is inappropriate for secondary hypertension because the estimate was substantially >1, suggesting that secondary hypertension in children and adolescents may be related to just a few genes.

Family history patterns can provide an initial estimation of genetic contribution and can offer insights for more detailed assessment of genetic patterns of diseases such as hypertension. Family history patterns are only the start to genetic understanding, however; more detailed genetic studies of hypertension in children and adolescents should prove valuable in defining populations amenable to intervention strategies.

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Appendix. Complete heritability calculation

Family History of Hypertension	Control	Affected	Total
No	na	nb	na + nb
Yes	nc	nd	nc + nd
	na + nc	nb + nd	na + nb + nc + nd

q_g = nc/(na + nc) = proportion of control patients with a family history of the disease

q_a = nd/(nb + nd) = proportion of affected patients with a family history of the disease

$p_g = 1 - q_g$

$p_a = 1 - q_a$

x_g = cutoff standard score for the proportion of the normal curve, $q_{g,1}$ Norminv(q_g) Norminv() is a function that returns a standard score from a normal distribution with mean = 0 and SD = 1 given the *P* value.

x_a = Cutoff standard score for the proportion of the normal curve, $q_{a,1}$. Norminv(q_a).

a_g = Standard score at the point where z (from Fig. 1) is the mean of the area represented by q_g; Norminv(1 − (.0039q_g^3 + .067 q_g^2 + .3903 q_g − .00003)). The area under the curve at the point where the height is z is not readily available from tables. The following curve is an approximation of the cumulative probability at that point.

b = slope = p_g * (x_g − x_a)/a

h^2 = 2b.

To calculate the standard error of h^2

ap_g = a (p_g − q_g)/q_g

$W_g = p_g/(a_g^2 * nc)$

$W_a = p_a/(a_a^2 * nd)$

Variance(h^2) = $(p_g/a_g - b * (ap_g - x_g))^2 * W_g + (p_g/a_g)^2 * W_a$

se (h^2)= 2* Variance$^{1/2}$

An Excel spreadsheet of the above formula is available from the author.

EXHIBIT 18

Clinical Therapeutics/Volume 30, Number 5, 2008

Letter to the Editor

Dear Dr. Walson:

In a 2004 publication, Balkrishnan et al[1] described a 24-month follow-up study in 1774 patients initiating antidiabetic drug therapy with pioglitazone (n = 1086) or rosiglitazone (n = 688) and reported no difference in total health care costs between the 2 drugs over the study period. This study was supported by Takeda Pharmaceuticals.

In 2007, Balkrishnan et al[2] described a study involving 1705 patients (1045 receiving pioglitazone and 660 receiving rosiglitazone) that was funded by GlaxoSmithKline. In this instance, the authors reported that when the observation period was extended from 24 to 30 months, rosiglitazone, the GlaxoSmithKline product, was associated with a 7.3% decrease in total health care costs relative to pioglitazone, the Takeda product.

Both studies employed the North Carolina Medicaid program database to identify patients who initiated therapy with either drug between July 1, 2001, and June 30, 2002. The logical assumption would be that the 2 studies used data from the same patients (with the exception of the 69 patients who were dropped when the observation period was extended from 24 to 30 months). Therefore, one might have expected the 2007 article ro comment on the dramatic effect on the study conclusions of a 6-month extension of the observation period. However, the 2007 article makes no reference to the 2004 article. In fact, the 2007 paper states that there had been no previous comparisons of the 2 drugs with respect to total health care costs.

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2. Balkrishnan R, Arondekar BV, Camacho FT, et al. Comparisons of rosiglitazone versus pioglitazone monotherapy introduction and associated health care utilization in Medicaid-enrolled patients with type 2 diabetes mellitus. *Clin Ther.* 2007;29:1306–1315.

EXHIBIT 19

Alleged Deviations Of Commonly Accepted Publication Practices By Lee and Mosely

Mosely and Lee worked together on research at The Ohio State University when Mosely's maiden was Sudimack. That research resulted in publications that, upon information and belief, appear to violate at least three commonly accepted practices within the scientific research community.

The publications evidencing these apparent violations are attached and include: Jennifer Sudimack, Wenjin Gua, Werner Tjacks, Robert Lee, *A Novel pH-Sensitive Liposome Formulation Containing Oleyl Alcohol, Biochimica et Biophysica Acta 1564 (2002)* ("Biochimica Publication"); Xing Pan, Huaquig Wang, Supriya Shukla, Masaru Sekido, Dianne Adams, Werner Tjacks, Rolf Barth, Robert Lee, *Boron-Containing Folate Receptor-Targeted Liposomes As Potential Delivery Agents For Neutron Capture Therapy, Bioconjugate Chem. 2002, 13,* ("Bioconjugate Publication"); Jennifer Sudimack, Dianne Adams, Joan Rotaru, Supiya Shukla, Junhua Yan, Masaur Sekido, Rolf Barth, Werner Jarks, Robert Lee, *Folate Receptor-Mediated Liposomal Delivery of A Lipophilic Boran Agent To Tumor Cells in Vitro For Neutron Capture Therapy, Pharmaeceutical Research, Vol. 19, No. 10, October 2002* ("Pharm/Res Publication"); Xing, Q. Pan, Huoqing Wang, Robert Lee, *Boron Delivery To A Murine Carcinoma Using Folate Receptor-Targeted Liposomes, Anticancer Research 22: 1629-1624 (2002)* publication by Lee ("Anti Cancer Publication"); Guangfeng Shi, Wenjin Guo, Stacy Stephenson, Robert Lee *Efficient Intercellular Drug and Gene Delivery Using Folate Receptor-Targeted pH-Sensitive Liposomes Composed Of Cationic/anionic Lipid Combinations, Journal Of Controlled Release 80 (2002)*("JCR Publication"); Michael Gosselin, Wenjin Guo, Robert Lee, *Incorporation of Reversibly Cross-Linked Polyplexes Into LPDII Vectors For Gene Delivery, Bioconjugate Chem. 2002, 13* ("Bioconjugate Publication II"); Wenjin Guo, Michael Gosselin, Robert Lee, *Characterization Of A Novel Diolein-Based LPDII Vector For Gene Delivery, Journal of Controlled Release 83 (2002)*)("JCR Publication II").

The first type of apparent violation involves duplicate publication issues related to six publications. Duplicate publication violations occur when two similar publications are published without referencing each other. Authors can avoid duplicate publication issues by "inform[ing] the editor of related manuscripts that the author has under editorial consideration or in press." *The American Chemical Society's Ethical Guidelines to Publication of Chemical Research (2006* ("ACS Publication Guidelines") p.3(emphasis added).

Editors may allow authors to republish previous publications provided the authors make a "full and prominent disclosure of its original source at the time of submission of the manuscript. *http://www.wame.org/resources/publication-ethics-policies-for-medical-journals (14 of 26), 6/8/2008 12:13:36 PM.* This disclosure must happen, "at the time of submission . . . even if . . . similar papers in press, and any closely related papers

previously published or currently under review at another journal." *Id.* (emphasis added); *See also, JACS Notice to Authors of Papers (2008)(*finding, "[w]hen related work by any of the authors is not available because it is in press (accepted), submitted, or in preparation for submission to *JACS* or another journal, a copy of each related paper should be uploaded as "Review-Only Material" at the time of submission for use by the reviewers and the Editors.")*(emphasis added)*.

It appears Mosely and/or Lee violated these rules in the Biochimica Publication and JCR Publication. This is because both articles address the same subject matter, used similar methods and contained similar writings. But, neither publication referenced or acknowledged the existence of the other.

The same thing happened in the Pharm/Res Publication and the Bioconjugate Publication. Both these publications addressed the subject matter, used similar methods and contained similar writings. Although the Pharm/Res Publication was published first, the Bioconjugate Publication did not reference the Pharm/Res Publication.

And, finally, the JCR Publication II and the Bioconjugate Publication II have the same authors, address same subject matter, and contain similar methods and content. Yet, neither publication referenced or acknowledged the existence of the other. Instead, Bioconjugate Publication II states, "[t]o our knowledge, this is the first report describing covalent stabilization of PEI/DNA polyplexes for subsequent inclusion in LPDII vectors." This statement appears false because the authors knew, or should have known, their JCR Publication II described and characterized the same polyplex systems.

Therefore, the authors' statement in the Bioconjugate Publication II evidences a second type of apparent publication violation – falsification. *See e.g.,* 42 CFR Parts 50 and 93, RIN 0940–AA04 (containing Dept. of Health and Human Services' Public Health Service Policies on Research Misconduct).

Moreover, the JCR Publication and the Biochimica Publication raise similar concerns. This is because the JCR Publication addresses generational pH sensitive liposome research also addressed in the Biochimica Publication. But, the Biochimica Publication claims the generation pH sensitive liposomes research detailed in the publication had not been previously published – even though it had been published in the JCR Publication.

The third apparent publication violation involves omission of negative data and/or inconsistent data publication. *See e.g.,* 42 CFR Parts 50 and 93, RIN 0940–AA04 (containing Dept. of Health and Human Services' Public Health Service Policies on Research Misconduct). For example, the Pharm/Res Publication and the Bioconjugate Publication both claimed folate receptor-targeted liposomes gave higher delivery of boron. But, Lee and Mosely knew, or should have known, of at least one case study and publication by Lee that called into question their folate delivery claims. *See, Anticancer Publication.* In failing to cite this negative data in the Pharm/Res Publication and the Bioconjugate Publication, Lee and Mosely appear to have violated commonly accepted practices regarding negative data.

A similar issue appears in the JCR Publication and the Biochimica Publication. This is because data discrepancies appear to exist within the two publications.

1044 *Bioconjugate Chem.* 2002, *13,* 1044–1053

Incorporation of Reversibly Cross-Linked Polyplexes into LPDII Vectors for Gene Delivery

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LPDII vectors are synthetic vehicles for gene delivery composed of polycation-condensed DNA complexed with anionic liposomes. In this study, we evaluated the stability and transfection properties of polyethylenimine (PEI, 25 kDa)/DNA polyplexes before and after covalent cross-linking with dithiobis(succinimidylpropionate) (DSP) or dimethyl·3,3′-dithiobispropionimidate·2HCl (DTBP), either alone or as a component of LPDII vectors. We found that cross-linking PEI/DNA polyplexes at molar ratios ≥10:1 (DSP or DTBP:PEI) stabilized these complexes against polyanion disruption, and that this effect was reversible by reduction with 20 mM dithioerythritol (DTE). Transfection studies with polyplexes cross-linked at molar ratios of 10:1−100:1 in KB cells, a folate receptor-positive oral carcinoma cell line, showed decreasing luciferase gene expression with increasing cross-linking ratio. Subsequently, polyplexes, cross-linked with DSP at a molar ratio of 10:1, were combined with anionic liposomes composed of diolein/cholesteryl hemisuccinate (CHEMS) (6:4 mol/mol), diolein/CHEMS/poly(ethylene glycol)-distearoylphosphatidylethanolamine (PEG-DSPE) (6:4:0.05 mol/mol), or diolein/CHEMS/folate-PEG-cholesterol (folate-PEG-Chol) (6:4:0.05 mol/mol) for LPDII formation. Transfection studies in KB cells showed that LPDII vectors containing cross-linked polyplexes mediated ~2−15-fold lower gene expression than LPDII prepared with un-cross-linked polyplexes, depending on the lipid:DNA ratio. Inclusion of PEG-DSPE at 0.5 mol % appeared to further decrease transfection levels ~2−5-fold. Compared with LPDII formulated with PEG-DSPE, LPDII incorporating 0.5 mol % folate-PEG-Chol exhibited higher luciferase activities at all lipid:DNA ratios tested, achieving an ~10-fold increase at a lipid:DNA ratio of 5. Compared with cross-linked LPDII vectors without PEG-DSPE, inclusion of folate-PEG-Chol increased luciferase acitivities 3−4-fold between lipid:DNA ratios of 1 and 5. Interestingly, inclusion of 1 mM free folate in the growth media during transfection increased transfection activity ~3−4-fold for cross-linked LPDII vectors and LPDII containing folate-PEG-Chol, but had no effect on the transfection activity of LPDII formulated with PEG-DSPE. However, in the presence of 5 mM free folate, the luciferase activity mediated by LPDII vectors containing folate-PEG-Chol was reduced ~6-fold. Transmission electron micrographs were also obtained to provide evidence of LPDII complex formation. Results showed that cross-linked LPDII vectors appear as roughly spherical aggregated complexes with a rather broad size distribution ranging between 300 and 800 nm.

INTRODUCTION

Nonviral delivery of gene therapy is an emerging strategy for the treatment of genetic disease, but progress is currently limited by inadequacies in the vectors available for gene transfer (*1*). The main deficiency of nonviral vectors is their relatively low in vivo gene transfer efficiency, compared with viral vectors. Therefore, research efforts have been focused on the design of nonviral vectors capable of achieving high tissue-specific gene expression with low immunogenicity and minimal toxicity. One particular strategy being evaluated to achieve these goals involves the use of LPD (liposome-entrapped polycation-condensed DNA) vectors containing covalently cross-linked polyplexes for gene delivery.

LPD vectors are synthetic vehicles for gene delivery composed of polycation-condensed DNA (polyplex) complexed with cationic or anionic liposomes via electrostatic interactions. Early LPD formulations, coined LPDI, utilized poly-L-lysine (PLL)-condensed DNA in association with cationic liposomes composed of dioleoylphosphatidylethanolamine/1-β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DOPE/DC-Chol; 6:4 mol/mol) (*2*). Subsequent alteration of the lipid component led to the development of LPDII transfection vectors. In contrast to LPDI, LPDII vectors are prepared with anionic pH-sensitive liposomes, such as dioleoylphosphatidylethanolamine/cholesteryl hemisuccinate (DOPE/CHEMS; 6:4 mol/mol) (*3, 4*). Since LPDII can be prepared with a net negative charge, these vectors have greater potential for receptor targeting, due to reduced nonspecific electrostatic interactions with nontarget cells. Moreover, neutral or negatively charged transfection vectors should be more compatible with the physiological environment of the systemic circulation, due to reduced interactions with components of the blood and decreased activation of the complement system (*5*).

First-generation LPDII vectors, those formulated with DOPE/CHEMS liposomes, exhibit nearly a complete loss of transfection activity in the presence of serum. Recently, our lab has developed a method to circumvent this limitation through incorporation of a novel anionic pH-sensitive liposome formulation composed of diolein/

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10.1021/bc025512c CCC: $22.00
Published on Web 08/20/2002

CHEMS (6:4 mol/mol) (*28*). Transfection studies with LPDII vectors containing diolein/CHEMS liposomes showed that transfection activity was sustained even when transfection was carried out in the presence of 50% fetal bovine serum (*28*). In addition, it has been shown that the DNA condensing agent can consist of polycations other than PLL, such as polyethylenimine (PEI) (*6*) or cationic dendrimers (*7*), without negatively affecting gene expression.

Recent efforts in our lab have been directed toward assessing the utility of homobifunctional cross-linking reagents for the development of reducibly cross-linked polyplexes for gene delivery (*8*). Two cross-linking reagents, dithiobis(succinimidylpropionate) (DSP) and dimethyl-3,3′-dithiobispropionimidate·2HCl (DTBP) (*9*), were utilized to create relatively high molecular weight polymers, composed of cross-linked low molecular weight PEI, for polyplex assembly. DSP is a homobifunctional *N*-hydroxysuccinimide (NHS) ester-based electrophilic cross-linking reagent containing an 8-atom spacer 12 Å in length. DTBP is similar to DSP in structure and length, but contains an imidoester reactive group for amidine bond formation. The significance of the amidine bond is that net molecular charge is retained after conjugation with DTBP, whereas amide bond formation mediated by DSP results in the elimination of two positive charges. Both cross-linking reagents react with primary amines to form stable covalent bonds, and are constructed around a centrally located disulfide linkage that is cleavable with common reducing agents after conjugation (Figure 1).

In the present study, we describe a novel LPDII transfection vector containing a covalently stabilized PEI-based polyplex core complexed with anionic pH-sensitive liposomes composed of diolein/CHEMS. We hypothesized that since LPDII formation is predicated on electrostatic interactions between a cationic polymer and plasmid DNA initially, and subsequently between cationic polyplexes and anionic liposomes, that covalent cross-linking of the polyplex core, after DNA condensation, would stabilize the polyplex against dissociation due to charge competition from the anionic lipid component. Therefore, studies were performed to assess the effects of cross-linking PEI/DNA polyplexes with DSP or DTBP on polyplex stability and transfection potential, and subsequently the transfection performance of LPDII vectors containing covalently cross-linked polyplexes in folate receptor-positive KB oral cancer cells in vitro. Furthermore, additional studies were perfomed to evaluate the effect of introducing a lipid-anchored folate receptor-targeting ligand or hydrophilic, poly(ethylene glycol) (PEG) coating into the LPDII formulation. Covalent attachment of folate for receptor targeting has proven to be effective in vitro for targeting liposomes (*10*–*12*) and LPDII vectors (*3*) to cancer cells which overexpress the high-affinity folate receptor. In addition, incorporation of PEG into liposome formulations has been shown to enhance tumor accumulation in vivo by reducing clearance by the reticuloendothelial system (RES), thus extending the circulation half-life (*13*, *14*).

MATERIALS AND METHODS

Materials. Cholesteryl hemisuccinate (CHEMS), di-9-octadecenoylglycerol (diolein, ~85% 1,3- and 15% 1,2-isomer), dithioerythritol (DTE), bovine serum albumin (BSA) protein standards, ethidium bromide (EtBr), and other general use chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Dithiobis(succinimidylpropionate) (DSP), dimethyl-3,3′-dithiobispropionimidate·2HCl (DTBP), and bicinchoninic acid (BCA) protein assay

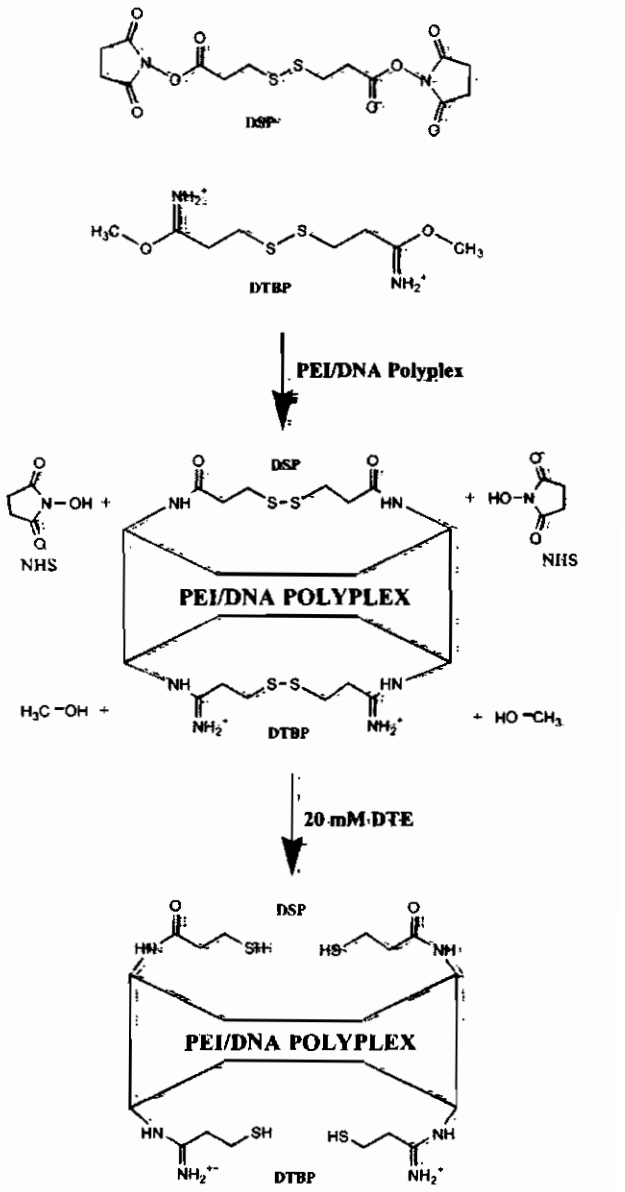

Figure 1. Proposed reaction scheme for covalent cross-linking of PEI/DNA polyplexes with cross-linking reagents DSP and DTBP. DSP cross-linking is illustrated above the schematic polyplex, while DTBP cross-linking is shown below. Also shown is the effect of disulfide bond reduction from 20 mM dithioerythritol (DTE).

reagents were purchased from Pierce (Rockford, IL). Anhydrous dimethyl sulfoxide (DMSO), polyethylenimine (PEI, branched, M_r ~25 000), and poly(methacrylic acid) (PMAA, M_w ~6500) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Luciferase assay reagents and recombinant luciferase were obtained from Promega (Madison, WI). All tissue culture media and supplies were purchased from Gibco BRL (Rockville, MD).

Plasmid Preparation. pcDNA3-CMV-Luc (pLuc, 7.1 kb) plasmid DNA encoding the firefly luciferase gene under control of the cytomegalovirus enhancer/promoter was obtained as a gift from Dr. Leaf Huang at the University of Pittsburgh School of Pharmacy. Cloning and preparation of plasmid DNA were performed by propagating transformed DH5-α *E. coli* in LB media containing 50 μg/mL ampicillin, followed by isolation and

purification with a commercially available plasmid purification kit (Qiagen, Valencia, CA). Concentration and purity of DNA were assessed spectrophotometrically by measuring absorbance at 260 and 280 nm (OD_{260}/OD_{280} ~ 1.7). Plasmid size and integrity were confirmed using agarose gel electrophoresis (0.9% agarose gel containing 0.5 μg/mL ethidium bromide) and cleavage with the restriction endonuclease *Hin*dIII (Gibco BRL).

Cell Culture. KB cells, a folate receptor-positive human oral squamous cell carcinoma, were obtained as a gift from Dr. Philip Low at Purdue University. The cells were cultured in folate-free RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 300 μg/mL L-glutamine at 37 °C in a 5% CO_2 humidified atmosphere. FBS provided the only source of folate in the growth media, which achieved physiologically relevant folate concentrations.

Liposome Preparation. Three pH-sensitive anionic liposome formulations were prepared: diolein/CHEMS (6:4 mol/mol); diolein/CHEMS/PEG (M_r ~2000)-distearoylphosphatidylethanolamine (DSPE) (6:4:0.05 mol/mol); and diolein/CHEMS/folate-PEG (M_r ~ 3350)-cholesterol (Chol) (6:4:0.05 mol/mol). PEG-DSPE was purchased from Avanti Polar Lipids, Inc., and folate-PEG-Chol was synthesized as described previously (*15*). Liposomes were prepared by mixing ~8−15 mg of total lipid (dissolved in chloroform as 20 mg/mL solutions) at the appropriate ratios in 13 × 100 mm glass test tubes. The chloroform was evaporated under a stream of nitrogen, and the lipids were further dried in a desiccator under vacuum for 30 min. The resulting lipid film was rehydrated with 20 mM HEPES buffer to a concentration of 3.33 mg/mL, and adjusted to pH 8.0−8.5. The suspensions were then vortexed and sonicated in a bath sonicator until the mean particle diameter was between 100 and 150 nm, as determined by dynamic light scattering using a NICOMP submicron particle sizer model 370. Lipid suspensions were stored at 4 °C until use.

Preparation of PEI/DNA Polyplexes and Stabilization by Cross-Linking. For polyplex preparation, 10 μg of pLuc DNA and the amount of PEI required to achieve the desired N/P (PEI nitrogen:DNA phosphate) ratios were each diluted in 250 μL of HEPES buffer (20 mM, pH 8.0). The PEI solution was quickly added to the DNA, and the resulting solution was then mixed and allowed to stand at room temperature (RT) for 15 min. For covalent cross-linking of polyplexes, DSP or DTBP, dissolved in anhydrous DMSO as a concentrated stock solution (1−10 mg/mL), was added to polyplexes at various concentrations depending on the desired cross-linking ratio. Cross-linking ratios are expressed as DSP: PEI molar ratios, and DMSO was kept below 5% of the total volume of polyplex solutions. After addition of the appropriate cross-linking reagent, samples were incubated at RT for 1 and 3 h for DSP and DTBP, respectively. Preparation of cross-linked and un-cross-linked polyplexes for transfection studies was performed similarly, with exceptions noted below.

Stability of Cross-Linked Polyplexes against Polyanion Disruption with PMAA. Polyplex stability against polyanion disruption was examined by measuring the ability of PMAA to restore DNA access to EtBr binding. Polyplexes with various N/P ratios containing 10 μg of pLuc DNA in 500 μL of HEPES buffer (20 mM, pH 8.0), either with or without DSP cross-linking, were diluted into 1.9 mL of 0.1× phosphate-buffered saline (PBS: 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). PMAA was added at various concentrations and incubated with polyplexes for 30 min at RT. To evaluate the reducibility of the disulfide bond present in the cross-linking reagent, 20 mM dithioerythritol (DTE) was added to some samples prior to the PMAA incubation. EtBr was present in the solution at a final concentration of 0.4 μg/mL, and EtBr fluorescence was measured on a Perkin-Elmer LS-50-B spectrometer, operated with a FTWinlab computer program (Morena Valley, CA). The excitation and emission wavelengths were set at 523 nm, with a 5 nm slit width, and 602 nm, with a 10 nm slit width, respectively.

Polyplex stability was further evaluated by agarose gel electrophoresis with EtBr staining. Polyplexes containing 1 μg of pLuc DNA prepared at a N/P ratio of 6, either with or without DSP cross-linking, were incubated with 100 μM PMAA for 30 min at RT. The reducibility of cross-linking was analyzed by including 20 mM DTE during the incubation. Half of each sample (0.5 μg of DNA) was then loaded on a 0.9% agarose gel containing 0.5 μg/mL EtBr, and run at 120 V for ~90 min.

Transfection of KB Cells with Cross-Linked Polyplexes or LPDII Vectors. For transfections, 10^5 KB cells/well were seeded in 24-well plates (Falcon) and allowed to incubate in folate-free RPMI 1640 growth media containing 10% FBS for approximately 20 h at 37 °C in a 5% CO_2 humidified atmosphere (~70−80% confluence). Immediately prior to transfection, cells were rinsed with warm PBS, and 0.5 mL of folate-free RPMI 1640 growth media was added to each well. LPDII transfections were carried out in the presence of 10% FBS, whereas polyplex transfections were performed under serum-free conditions. Transfection complexes were added to each well and incubated with KB cells for 2 or 4 h for LPDII vectors or polyplexes, respectively. After rinsing the cells with 300 μL of warm PBS, the transfection medium was replaced with 0.5 mL of fresh RPMI containing 10% FBS. A further 24 h incubation was imposed, followed by cell lysis and analysis of luciferase gene expression (see below).

PEI/pLuc polyplexes (N/P = 10) utilized for transfection were prepared as described above for EtBr fluorescence analysis, but with 1 μg of DNA and the desired amount of PEI, each dissolved in 30 μL of 20 mM HEPES buffer prior to mixing. Various concentrations of DSP or DTBP, dissolved in DMSO, were then added to polyplex solutions to achieve different cross-linking ratios, and incubated at RT for 1 or 3 h, respectively. DMSO comprised less than 5% of the solution volume.

LPDII vectors, containing PEI/pLuc polyplexes (N/P = 4) and diolein/CHEMS liposomes, were prepared as described above by diluting 3 μg of DNA and the desired amount of PEI, each in 50 μL of HEPES buffer (20 mM, pH 8.0) prior to mixing. For cross-linking, 2.5 μL of DSP in DMSO (0.1 mg/mL) was added to each sample, lightly vortexed, and incubated at RT for 1 h. Various concentrations of diolein/CHEMS liposomes in 50 μL of HEPES buffer were then added to each sample, depending on the desired lipid:DNA ratio, and allowed to stand at RT for 15 min. Finally, 50 μL (1 μg of DNA/well) of the resulting solution was added to each well of a 24-well plate to evaluate transfection potential.

To analyze luciferase expression, the growth medium was removed from each well, and the cells were rinsed twice with cold PBS. Next, 200 μL of ice-cold lysis buffer (0.5% Triton X-100, 100 mM Tris-HCl, 2 mM EDTA, pH 7.8) was added to each well, followed by incubation on ice for 30 min. The lysate was then removed and centrifuged for 1 min at 13 000 rpm. Finally, 10 μL of cellular lysate was mixed with 50 μL of luciferase substrate (Promega), and the luminescence was measured for 10 s on a Mini-Lum luminometer (Bioscan, Inc.,

Washington, DC). Relative light units were standardized for protein concentration as determined by the BCA protein determination assay using bovine serum albumin standards. Luciferase activity is also expressed as femtomoles of luciferase per milligram of protein, determined from a standard curve of luciferase activity using recombinant luciferase.

Negative Stain Transmission Electron Microscopy (TEM). DSP cross-linked polyplexes (10:1) and LPDII particles were prepared as described above at an N/P ratio of 6 and a lipid:DNA ratio of 5 (w/w), respectively, with each sample containing 10 μg of plasmid DNA. An aqueous solution of bacitracin was prepared at a concentration of 200 mg/mL, and diluted to a working strength of 10 mg/mL. An aqueous 1% uranyl acetate solution was also prepared, and was filtered and centrifuged to remove any particulate matter before use. Samples were prepared by placing a 200 mesh size Formvar-coated copper grid on a drop of bacitracin solution (10 mg/mL) for 1 min, before wicking away excess solution from the grid with filter paper. Next, each grid was placed in contact with the appropriate sample for 3 min, and the excess liquid was again wicked away with filter paper. The grid was then subjected to two brief sequential rinses by placing the grid on a drop of water and wicking away excess fluid. Finally, the grid was incubated in 1% uranyl acetate solution for 2.5 min, excess liquid was removed, and the grid was allowed to dry at RT for 5 min. Electron micrographs were obtained at 60 kV at 100000× magnification for polyplexes and diolein/CHEMS liposomes or 45000× magnification for LPDII particles.

RESULTS

To evaluate the effect of cross-linking on polyplex-mediated transfection of KB cells in vitro, 25 kDa PEI/pLuc DNA polyplexes were prepared at an N/P (PEI nitrogen:DNA phosphate) ratio of 10, followed by cross-linking with DSP or DTBP at various cross-linker:PEI molar ratios, as described under Materials and Methods. Specifically, cross-linking ratios of 0, 10, 20, 50, and 100:1 were evaluated. An N/P ratio of 10 was chosen based on previous studies showing efficient gene expression at this ratio (*16*). Figure 2 shows that as the cross-linking ratio with DSP or DTBP increases from 0 (control) to 100:1, luciferase gene expression decreases by approximately 3−4 orders of magnitude. The data further show that transfection is relatively independent of the reagent used for cross-linking, with nearly identical luciferase activity at a cross-linking ratio of 10. However, at higher cross-linking ratios (≥20), slightly better gene expression was observed with polyplexes cross-linked with DTBP.

Our next goal was to evaluate the stability of polyplexes, either with or without covalent stabilization by cross-linking, in the presence of a competing polyanion. Our initial stability analysis was performed on conventional PEI/DNA polyplexes prepared at a variety of N/P ratios, namely, 0 (DNA only), 2, 3, 5, and 10. These polyplexes were exposed to various concentrations of poly(methacrylic acid) (PMAA), an organic polyanion, ranging from 0 to 100 μM, followed by addition of ethidium bromide (EtBr) and quantitative fluorescence measurements. Detection of EtBr fluorescence is an indication of DNA being accessible for EtBr binding due to polyplex dissociation, or at least local decondensation of DNA. The

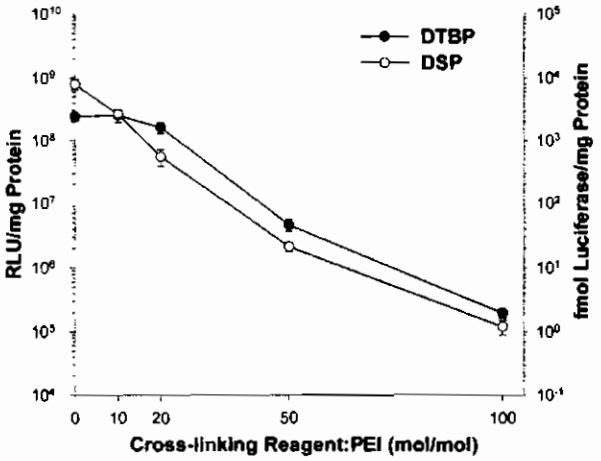

Figure 2. Effect of reducible cross-linking on the transfection activities of PEI/DNA polyplexes. Polyplexes were prepared at an N/P ratio of 10. Polyplexes were then cross-linked with DSP or DTBP at cross-linker:PEI molar ratios of 0, 10, 20, 50, and 100:1. The transfection activity of the covalently cross-linked polyplexes was then evaluated in KB cells in vitro. Cells were exposed to transfection vectors for 4 h in serum-free media, and luciferase activity was assayed 24 h later.

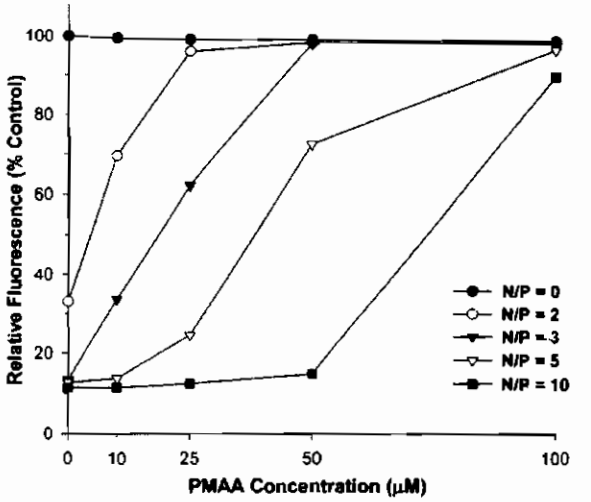

Figure 3. EtBr fluorescence from interactions with PEI/DNA polyplexes in the presence of a competing polyanion (PMAA). Polyplexes were prepared at N/P ratios of 0, 2, 3, 5, and 10, and exposed to various concentrations of PMAA up to 100 μM. EtBr was added to each sample at a concentration of 0.4 μg/mL, and fluorescence was measured at excitation and emission wavelengths of 523 and 602 nm, respectively.

effect on fluorescence (N/P = 0). The results also show that in the absence of PMAA, EtBr fluorescence for all polyplexes decreases to ~10% of control, with the exception of polyplexes prepared at an N/P ratio of 2. Polyplexes prepared at this ratio are known to cause incomplete condensation of DNA due to inadequate charge neutralization, which led to an EtBr fluorescence reading of ~30%. As the concentration of PMAA was increased, progressively higher fluorescence readings were observed for all polyplexes, with values eventually approaching 100% of control at a PMAA concentration of 100 μM.

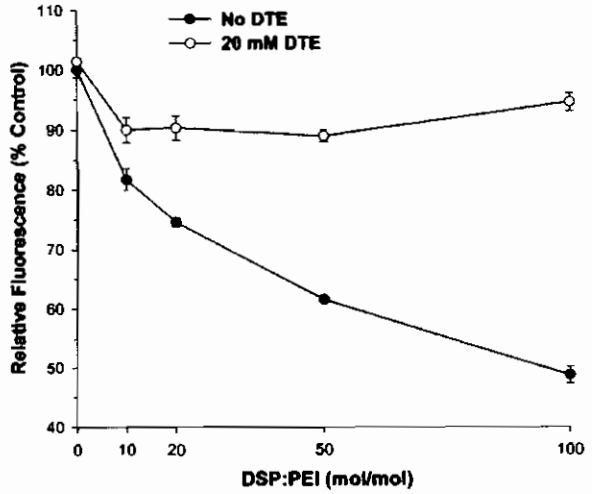

Figure 4. EtBr fluorescence from interactions with DSP cross-linked PEI/DNA polyplexes in the presence of 100 μM PMAA, with or without disulfide bond reduction with DTE. Polyplexes were prepared at a N/P ratio of 6, and cross-linked with DSP at DSP:PEI molar ratios of 0, 10, 20, 50, and 100:1. Cross-linked polyplexes were then exposed to 100 μM PMAA for 30 min, with or without 20 mM DTE. EtBr was added to each sample at a concentration of 0.4 μg/mL, and fluorescence was measured at excitation and emission wavelengths of 523 and 602 nm, respectively.

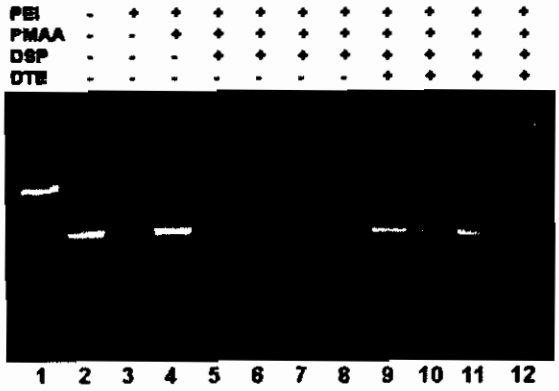

Figure 5. Stability of covalently cross-linked polyplexes analyzed by agarose gel electrophoresis. Polyplexes were prepared at a N/P ratio of 6, and cross-linked with DSP at DSP:PEI molar ratios of 10, 20, 50, and 100:1. Cross-linked polyplexes were then exposed to 100 μM PMAA for 30 min, with or without 20 mM DTE, and analyzed by agarose gel electrophoresis (0.5 μg of DNA/lane). Lane 1 is *Hin*dIII-digested phage λ DNA, used as a size marker; lane 2 is pLuc DNA only; lane 3 is PEI/pLuc polyplex (N/P = 6); lane 4 is PEI/pLuc polyplex with 100 μM PMAA; lanes 5–8 are PEI/pLuc polyplexes cross-linked with DSP at 10:1, 20:1, 50:1, and 100:1, respectively, with 100 μM PMAA; lanes 9–12 are identical to lanes 5–8, except for the addition of 20 mM DTE.

Since polyplex stability is apparently compromised by the presence of a competing polyanion, experiments were then performed to evaluate the consequence of cross-linking PEI/DNA polyplexes with DSP, at various DSP:PEI molar ratios, before exposure to PMAA. Polyplexes were prepared at an N/P ratio of 6, and were then cross-linked with DSP at molar ratios of 10, 20, 50, and 100:1. PMAA was included in all samples at a concentration of 100 μM, since that concentration restored nearly 100% EtBr fluorescence when incubated with un-cross-linked polyplexes. As seen in Figure 4, the results show that as the DSP:PEI cross-linking ratio is increased, PEI/DNA polyplexes become increasingly resistant to polyanion challenge, exhibited by the decrease in EtBr fluorescence. In fact, fluorescence readings for polyplexes cross-linked at a ratio of 100:1 decreased by more than 50%. However, when 20 mM dithioerythritol (DTE) was introduced to promote reduction of the intramolecular disulfide bond present in DSP, effectively reversing stabilization, EtBr fluorescence was restored to ~90% of control for all samples.

To obtain visual evidence, to supplement the quantitative fluorescence readings in Figure 4, agarose gel electrophoresis was performed. The underlying principle was the same as for fluorometric analysis (i.e., EtBr fluorescence is restored upon polyplex dissociation or local decondensation of DNA); however, visualization by electrophoresis provides a basis to discern between dissociation or local DNA decondensation. As seen in lane 4 of Figure 5, incubation of un-cross-linked polyplexes (N/P = 6) with 100 μM PMAA results in polyplex dissociation, as evidenced by the DNA migration through the gel. The results also show (lanes 5–8) that despite DSP cross-linking, some EtBr fluorescence can be seen in the loading wells, with the intensity slightly decreasing as the cross-linking ratio is increased. Lanes 9–12 illustrate the effect of DTE reduction of disulfide bonds on DNA migration and EtBr fluorescence. At cross-linking ratios of 10, 20, and 50:1 (lanes 9–11), pLuc DNA regained its fluorescence and was free to migrate through the gel. visible in the loading well, with a faint band appearing where plasmid DNA would be expected, despite DTE reduction.

Transfection studies were performed to compare the transfection performance between LPDII vectors either with or without covalent cross-linking of polyplexes in KB cells in vitro. Polyplexes were prepared at an N/P ratio of 4, yielding condensed particles with a slight positive charge. Where DSP cross-linking was employed, polyplexes were cross-linked at a molar ratio of 10:1 (DSP:PEI). Figure 6 displays the transfection results from a series of four different LPDII formulations, along with results from a 1 mM free folate competition study for the folate receptor-targeted formulation. Specifically, the formulations tested included conventional diolein/CHEMS LPDII particles without DSP cross-linking, LPDII including covalently cross-linked polyplexes, LPDII prepared with PEG containing liposomes, and receptor-targeted LPDII particles containing a lipid-anchored folate moiety. As indicated, all polyplexes were PEI/DNA complexes, and the anionic liposome component was composed of diolein/CHEMS prepared at a molar ratio of 6:4. Inclusion of PEG-DSPE, for steric stabilization, or folate-PEG-Chol, for receptor targeting, was achieved by adding 0.5 mol % of the appropriate lipid-anchored molecule during preparation of the diolein/CHEMS liposomes.

Figure 6A,B depicts the luciferase gene expression achieved with each LPDII formulation as a function of lipid:DNA (w/w) ratio. Transfection levels mediated by all formulations increased initially, before achieving peak luciferase activities at a lipid:DNA ratio of 2.5. All formulations then exhibited a subsequent decrease in luciferase activity at a lipid:DNA ratio of 5. At ratios beyond 5, luciferase activities appeared to level off, with the exception of LPDII prepared without DSP cross-linking, which displayed steadily decreasing luciferase activities with increasing lipid:DNA ratio. From a comparison of conventional diolein/CHEMS LPDII vectors and LPDII that include DSP cross-linked polyplexes, it can be seen that cross-linking at a ratio of 10:1 led to an ~16-fold decrease in gene expression at lipid:DNA

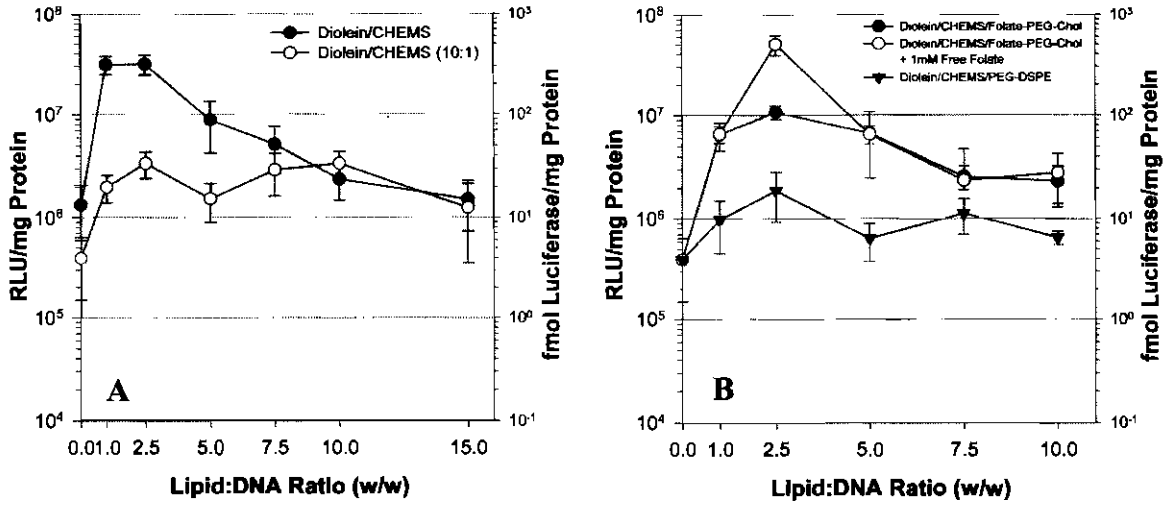

Figure 6. Transfection of KB cells with various LPDII formulations as a function of lipid:DNA ratio. The lipid:DNA ratios tested were 0 (cross-linked or un-cross-linked polyplex alone), 2.5, 5, 7.5, 10, and 15. All formulations contain PEI/pLuc DNA polyplexes prepared at a N/P ratio of 4. Except where noted in the figure, all polyplexes were cross-linked with DSP at a DSP:PEI molar ratio of 10:1. Diolein/CHEMS liposomes were prepared at a molar ratio of 6:4, and where indicated, 0.5 mol % PEG-DSPE or folate-PEG-Chol was included.

ratios ≤7.5, but that similar luciferase activities were achieved at ratios beyond 10. Inclusion of PEG-DSPE at 0.5 mol % appeared to consistently hinder transfection levels, reducing luciferase activities ~2−5-fold, compared to LPDII containing cross-linked polyplexes prepared without PEG-DSPE. In contrast, LPDII containing folate-PEG-Chol exibited higher gene expression at all lipid:DNA ratios tested, compared with LPDII vectors containing PEG-DSPE. In fact, luciferase activities for LPDII containing folate-PEG-Chol were ~5−10 times greater than the PEG-DSPE formulation at lipid:DNA ratios between 1 and 5, with decreasing differences at higher ratios. Compared with cross-linked LPDII without PEG-DSPE, inclusion of 0.5 mol % folate-PEG-Chol increased luciferase acitivities 3−4-fold between lipid:DNA ratios of 1 and 5, and, again, led to decreasing differences at high lipid:DNA ratios. Inclusion of 1 mM free folate in the growth media during transfection with LPDII vectors containing folate-PEG-Chol led to luciferase activities nearly identical to those achieved in the absence of free folate, with the exception of an ~4.5-fold increase in activity at a lipid:DNA ratio of 2.5.

In contrast to the observed results, luciferase activities were expected to decrease for LPDII containing folate-PEG-Chol in the presence of 1 mM free folate, due to competition for the folate receptor. However, it has been shown previously that addition of free folate to the growth media during transfection increases transfection levels mediated by PEI/DNA polyplexes (*17*). Therefore, transfection studies to compare the effect of free folate on gene expression were repeated with conventional diolein/CHEMS LPDII vectors, LPDII formulated with PEG-DSPE, and LPDII containing folate-PEG-Chol, all containing DSP cross-linked polyplexes at a ratio of 10:1 (DSP:PEI). Free folate was included in the growth media at a concentration of 1 mM, as in earlier transfection studies, with additional concentrations of 2 and 5 mM included for the folate receptor-targeted formulation. Figure 7 shows that inclusion of 1 mM free folate in the growth media increased luciferase activities 3−4-fold for diolein/CHEMS LPDII and LPDII containing folate-PEG-Chol, but had little effect on LPDII containing PEG-DSPE. Furthermore, increasing the free folate concen-

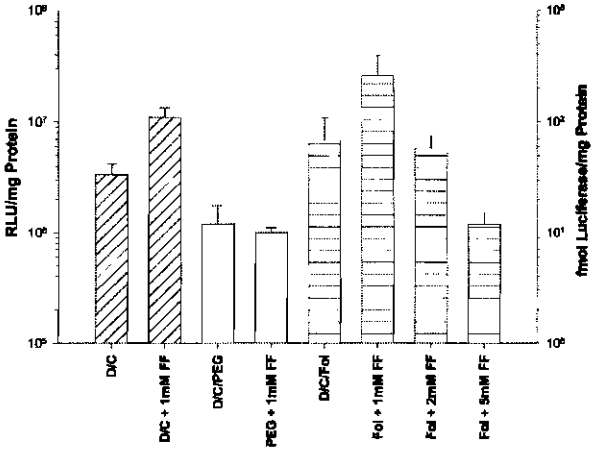

Figure 7. Effect of free folate on the transfection activity of the LPDII formulations containing DSP cross-linked polyplexes. Free folate (FF) was included at 1, 2, or 5 mM in the growth media during transfection. All polyplexes used in LPDII preparation were cross-linked with DSP at a DSP:PEI molar ratio of 10:1. The three LPDII formulations tested include diolein/CHEMS (D/C), diolein/CHEMS/PEG-DSPE (D/C/PEG or PEG), and diolein/CHEMS/folate-PEG-Chol (D/C/Fol or Fol). Diolein/CHEMS liposomes were prepared at a molar ratio of 6:4, and where indicated, 0.5 mol % PEG-DSPE or folate-PEG-Chol was included.

targeted LPDII vectors decreased luciferase activity, in comparison to 1 mM free folate, and achieved similar transfection levels to those observed in the absence of free folate. Increasing the free folate concentration to 5 mM led to a further decrease in luciferase activity, which was ~6-fold lower than transfection levels obtained without free folate present in the growth media.

Finally, negative stain transmission electron microscopy was performed in order to obtain evidence confirming the association between diolein/CHEMS liposomes and cross-linked polyplexes. Samples were prepared as described under Materials and Methods, and the results are illustrated in Figure 8. Panel A shows PEI/DNA polyplexes cross-linked with DSP at a molar ratio of 10:

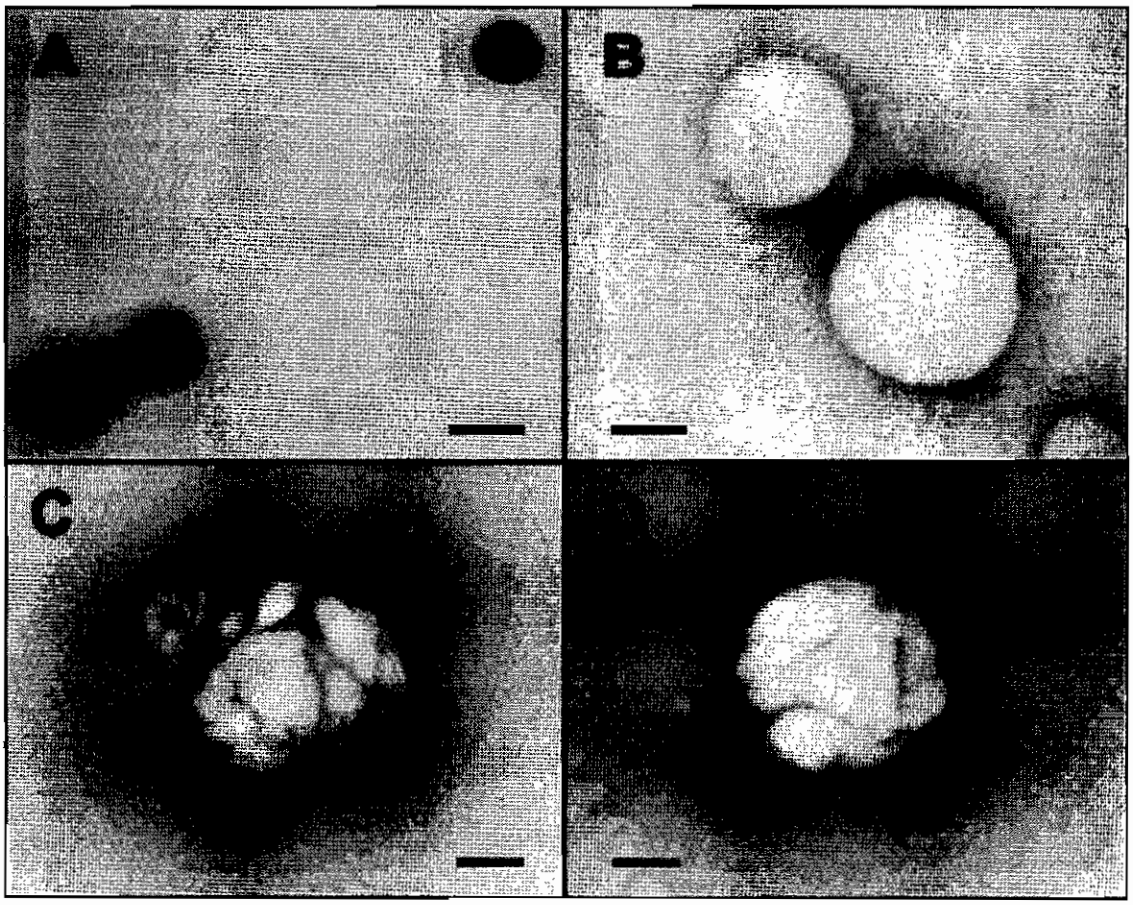

Figure 8. Negative stain transmission electron microscopy. DSP cross-linked polyplexes (10:1) and LPDII particles were prepared at an N/P ratio of 6 and a lipid:DNA ratio of 5 (w/w), respectively, with each sample containing 10 μg of plasmid DNA. Electron micrographs were obtained at 60 kV. (Panel A) DSP cross-linked PEI/DNA polyplexes at 100000× magnification. (Panel B) Diolein/CHEMS liposomes (6:4, mol/mol) at 100000× magnification. (Panels C and D) DSP cross-linked LPDII vectors at 45000× magnification. In panels A and B, the bar = 100 nm; in panels C and D, the bar = 200 nm.

ranging in diameter from ~70 to 100 nm. Panel B shows diolein/CHEMS liposomes prepared at a molar ratio of 6:4, which appeared as white spherical particles having a diameter between 100 and 250 nm. Finally, panels C and D depict LPDII particles formed at a lipid:DNA ratio of 5. These complexes were manifested as aggregated structures, roughly spherical in shape, with diameters ranging between 300 and 800 nm.

DISCUSSION

Currently, the primary limitation in the development of clinically useful nonviral gene therapy protocols is the lack of safe and efficient nonviral vectors for in vivo gene delivery. Here, we evaluate a novel approach to improve the stability and transfection potential of polyplex- and LPDII-mediated gene transfer. We have shown that cross-linking PEI/DNA polyplexes stabilizes these complexes against polyanion disruption, and that cross-linking is reversible upon reduction by DTE. We further demonstrated that LPDII transfection vectors mediate variable levels of transfection, depending on the presence of DSP cross-linking and the incorporation of PEG-DSPE or folate-PEG-Chol as a component of the diolein/CHEMS liposomes used for LPDII formation. It was also observed that the presence of 1 mM free folate in the transfection media has a surprisingly beneficial effect on transfection, but that this effect was dose-dependent for LPDII vectors containing folate-PEG-Chol. Finally, we provide evidence, by way of electron micrographs, that diolein/CHEMS liposomes and DSP cross-linked polyplexes form aggregated complexes under our experimental conditions.

Initial transfection studies with cross-linked polyplexes were performed to assess the effects of DSP or DTBP cross-linking of PEI/DNA polyplexes on luciferase reporter gene expression. Branched, 25 kDa PEI was used as the condensing agent because previous studies indicate that PEI is an effective transfection agent both in vitro (*16, 18*) and in vivo (*19*−*21*). Transfection results indicated that cross-linking polyplexes with DSP or DTBP, after DNA condensation, leads to a reduction in gene expression in KB cells, with significantly lower luciferase activities (3−4 orders of magnitude) observed at higher cross-linking ratios. These results were not surprising, since increasing the cross-linking ratio would be expected to increase polyplex stability. While increased stability may be beneficial prior to cellular uptake, ultimately the DNA has to be accessible by the nuclear transcription machinery, thus mandating polyplex dissociation and DNA decondensation preceding gene expression. High cross-linking ratios would require more extensive intracellular reduction of disulfide bonds present in the cross-linking reagents in order to reverse stabilization, which may ultimately limit gene expression.

Comparing the two cross-linking reagents, we found transfection activity to be slightly higher with DTBP cross-linked polyplexes, at cross-linking ratios ≥20.

However, in our experience, DTBP is less reactive toward PEI, compared with DSP, under the chosen reaction conditions. The slightly greater transfection levels mediated by DTBP cross-linked polyplexes at higher cross-linking ratios may be a reflection of less extensive cross-linking, and consequently a lower reduction in gene expression. Alternatively, the retention of positive charge after DTBP cross-linking may promote more efficient gene transfer. In the end, DSP was used to cross-link polyplexes for inclusion in LPDII vectors because cross-linking with DSP can be accomplished with a shorter reaction time, and it is possible to monitor the appearance of NHS from DSP spectrophotometrically to gauge reaction progress. In addition, there were relatively insignificant differences in transfection levels between DSP and DTBP cross-linked polyplexes, especially at a cross-linking ratio of 10:1.

While it was important to understand the effect of cross-linking on transfection levels, it was equally important to investigate the stability of cross-linked polyplexes, to justify covalent stabilization. Therefore, polyplex stability was evaluated in the presence of a competing polyanion, namely, PMAA, which should mimic the effect of polyanionic liposomes on polyplex integrity during LPDII formation. Anionic liposomes themselves were not used as the competing polyanion because lipid concentrations utilized for LPDII formulation interfered with EtBr fluorescence detection. Initially, we were interested in determining whether un-cross-linked polyplexes were susceptible to dissociation from polyanion disruption as a function of N/P ratio. Therefore, polyplexes were prepared at several N/P ratios, and incubated with various PMAA concentrations, followed by EtBr fluorescence analysis. This analysis is based on the concept that a large increase in fluorescence is observed upon intercalation of DNA by the phenanthridium moiety of EtBr (*22*). Condensed DNA, like the DNA present in polyplexes prepared with a net positive charge, will exclude EtBr from intercalating between base pairs, thus providing minimal fluorescence. In contrast, uncondensed DNA, resulting from local decondensation of DNA or polyplex dissociation, will permit EtBr intercalation to yield a sharp increase in EtBr fluorescence. The increase in fluorescence should be proportional to the amount of DNA available for intercalation. Our results showed that polyplexes exposed to PMAA exhibit an increase in EtBr fluorescence, and that fluorescence was directly related to PMAA concentration and inversely related to the N/P ratio. This result suggests that conventional polyplexes are susceptible to polyanion disruption, and that polyplexes prepared with large N/P ratios are more stable than those prepared with low ratios.

We anticipated that reversible cross-linking would stabilize polyplexes, and that reduction of the intramolecular disulfide bond present in DSP would restore polyplex susceptibility to polyanion disruption. Therefore, we cross-linked PEI/DNA polyplexes at various DSP:PEI molar ratios and evaluated EtBr fluorescence in the presence of 100 μM PMAA, either with or without 20 mM DTE. The results showed that increasing the cross-linking ratio leads to a decrease in EtBr fluorescence, suggesting more stable polyplexes at higher cross-linking ratios. Reduction with 20 mM DTE was found to restore fluorescence levels to ~90% of control. While these results are useful in demonstrating covalent stabilization with evidence regarding the interaction between polyplexes and diolein/CHEMS liposomes. Nevertheless, although these results were considered positive in terms of demonstrating stabilization and cross-linking reversibility, they also introduced a dilemma in choosing the appropriate cross-linking ratio of polyplexes for incorporation into LPDII transfection particles. On one hand, earlier transfection results with cross-linked polyplexes showed a minimal reduction in gene expression if the cross-linking ratio was relatively low. However, the stability results showed that EtBr fluorescence was still ~75−80% of control for complexes cross-linked to the same extent. Therefore, the degree by which DSP might have contributed to cross-linking stability was unclear.

Consequently, polyanion disruption of cross-linked polyplexes was repeated as described above, but the samples were instead visualized by agarose gel electrophoresis. This assay provided a basis to distinguish between polyplex dissociation and local decondensation of DNA. With polyplex dissociation, free plasmid DNA would be expected to migrate through the gel at a rate proportional to its size, but with local decondensation of DNA, fluorescence would likely be observed in the loading well. This stems from the fact that DNA migration through the agarose gel is based on electrostatic attraction between polyanionic DNA and a positively charged anode. However, when DNA is present in the form of a polyplex, the negative charge is neutralized by the cationic polymer, consequently precluding its migration through the gel. In addition, the size increase resulting from the presence of the high molecular weight PEI in the polyplex would further hinder DNA migration. Ultimately, the electrophoresis results suggested that low cross-linking ratios are adequate to prevent dissociation, but will not completely prevent local DNA decondensation. This was shown in Figure 5 by DNA migration with un-cross-linked polyplexes in the presence of 100 μM PMAA (lane 4), but after cross-linking with DSP, fluorescence was only visible in the loading wells (lanes 5−9). These results provided a logical basis for cross-linking polyplexes at a DSP:PEI molar ratio of 10:1 for incorporation into LPDII vectors. Specifically, this ratio was chosen because preliminary transfection results using polyplexes cross-linked at 10:1 showed a minimal reduction in gene expression, compared with un-cross-linked polyplexes, and agarose gel analysis further showed that these complexes remained associated even with relatively strong polyanion challenge (100 μM PMAA).

To delineate the effects on gene expression, the transfection potential of four related LPDII vectors was evaluated in KB cells in vitro. PEI/DNA polyplexes were formed at an N/P ratio of 4, and, where indicated, cross-linked with DSP at a DSP:PEI molar ratio of 10:1. An N/P ratio of 4 was utilized because this ratio mediates complete condensation of plasmid DNA to form positively charged complexes, yet minimizes free PEI in solution not participating in polyplex formation (*19*, *23*). If free PEI were present in the solution after polyplex formation, DSP could react preferentially with free PEI and reduce polyplex stabilization by consuming DSP. The anionic liposomes used for LPDII formation were composed of a novel serum-resistant formulation consisting of diolein/CHEMS (6:4 mol/mol). The four LPDII formulations tested for luciferase reporter gene expression included

From the transfection results shown in Figure 6A, the first comparison can be made between LPDII vectors containing DSP cross-linked polyplexes and those without cross-linking. The transfection results show that LPDII vectors with covalent cross-linking mediated lower luciferase activities at lipid:DNA ratios ≤7.5, yet similar transfection levels at lipid:DNA ratios ≥10. Results from the transfection studies using cross-linked polyplexes for gene transfer, illustrated in Figure 2, showed that DSP cross-linking inhibited gene expression, and this effect may contribute to the observed decrease in transfection activity mediated by LPDII vectors containing cross-linked polyplexes. Alternatively, LPDII particle formation may be deleteriously altered by the presence of DSP, or more likely by the elimination of positive charges on PEI after reaction with DSP, since LPDII particles largely depend on electrostatic attraction for complex formation. LPDII vectors prepared in the absence of cross-linking achieve a peak luciferase activity at a lipid:DNA ratio of 2.5, and then display steadily decreasing transfection levels. In contrast, LPDII containing cross-linked polyplexes display a more level luciferase activity profile over the entire range of lipid:DNA ratios. This observation suggests that particle assembly may be saturable at low lipid:DNA ratios, again possibly due to the elimination of positive charge after DSP cross-linking. Despite the disparity in transfection activity between these two formulations, LPDII particles containing cross-linked polyplexes did mediate significant levels of transfection, and, therefore, may prove useful under in vivo conditions where complex stability could be a more dominant factor.

Inclusion of PEG-DSPE at 0.5 mol % into the LPDII formulation containing DSP cross-linking resulted in a further reduction in gene expression at all lipid:DNA ratios tested, compared with the cross-linked formulation in the absence of PEG-DSPE. This effect may be the result of reduced interaction between polyplexes and liposomes, or a reduction in cellular uptake. Since particle size is an important determinant for in vitro transfection, with smaller particles achieving lower transfection, it could also be postulated that incorporation of PEG-DSPE reduces any interactions between transfection complexes in solution that might cause an increase in particle size, thus reducing gene expression.

The contribution of folate receptor targeting, through incorporation of 0.5 mol % folate-PEG-Chol, can be seen in Figure 6B by comparing the transfection activity of folate receptor-targeted LPDII vectors with LPDII formulated with PEG-DSPE. Based on that comparison, the data suggest that attachment of folate to the distal end of a PEG linker does mediate an increase in luciferase activity at all lipid:DNA ratios, but especially at ratios ≤5. However, to confirm that folate receptor-mediated endocytosis contributes to increased gene expression, a folate receptor competition study was performed by introducing 1 mM free folate in the growth media during transfection. The results, also shown in Figure 6B, were inconsistent with our expectations that luciferase activity would decrease in the presence of competition by free folate. However, as indicated earlier, previous studies in our lab have shown that inclusion of free folate in the growth media during transfection increases the transfection activity of PEI-based polyplexes. In effect, transfection studies were performed to determine if free folate had a similar effect on LPDII particles prepared at a lipid:DNA ratio of 2.5. We found that inclusion of 1 mM free folate in the growth media during transfection increased the transfection activity of LPDII containing cross-linked polyplexes and LPDII containing folate-PEG-Chol, but had little effect on the transfection activity of LPDII formulated with PEG-DSPE. To determine if higher concentrations of free folate could inhibit the transfection activity of folate receptor-targeted LPDII vectors, similar transfection studies were performed in the presence of 2 and 5 mM free folate. The results showed that luciferase activity was inversely related to the concentration of free folate in the growth media. The mechanism responsible for the increased gene expression in the presence of 1 mM free folate has not been determined, and it is curious that LPDII containing PEG-DSPE were relatively unaffected. Regardless, we conclude that inclusion of folate-PEG-Chol in LPDII vectors mediates greater luciferase activities compared with LPDII formulated with PEG-DSPE. Furthermore, the presence of 1 mM free folate in the growth media during transfection apparently has a beneficial effect on transfection activity, and, therefore, does not offer conclusive evidence that increased gene expression from inclusion of folate-PEG-Chol is truly receptor-mediated. The fact that higher concentrations of folate do reduce observed transfection levels is consistent with expectations, but 5 mM free folate is orders of magnitude greater than the K_d for folic acid, $10^{-9}-10^{-10}$ M. Although the concentrations cited below are ~10-times lower, similar observations have been reported previously that show the IC_{50} for folate receptor-targeted liposomes is 0.2−0.4 mM (*10*, *15*). The high binding strength of these liposomes has been attributed, in part, to the high binding affinity achieved by multivalent attachment of multiple targeting ligands.

Finally, negative stain transmission electron micrographs of DSP cross-linked polyplexes, diolein/CHEMS liposomes, and LPDII vectors containing covalently cross-linked polyplexes were obtained to gather size and structural information, and to provide evidence of LPDII complex assembly. The images shown in Figure 8A display cross-linked polyplexes that appear as dark spherical particles having a diameter between 70 and 100 nm. This micrograph is consistent with previously published observations of PEI-based polyplexes (*24*, *25*). Figure 8B displays images of diolein/CHEMS liposomes, which appear as white spherical particles smaller than 250 nm. To our knowledge, these are the first published images of diolein/CHEMS liposomes. Figure 8C,D portrays LPDII particles composed of diolein/CHEMS liposomes and cross-linked polyplexes. The diameter of these particles was somewhat variable, but was generally larger than either the polyplexes or the liposomes. The LPDII particles displayed in Figure 8 had diameters between 500 and 700 nm. This size information suggests that the increased transfection performance of LPDII particles may in part be a result of increased particle size, which would promote sedimentation of transfection particles in vitro. In addition, these images appear consistent with the hypothesis that LPDII complexes are composed of aggregated complexes of polyplex and anionic liposomes. However, since the polyplex would theoretically be located inside this complex, and the LPDII particles were not visualized in cross section, it cannot be ruled out that the particles observed in Figure 8C,D are simply aggregates of liposomes. The fact that these types of particles were not observed when viewing diolein/CHEMS liposomes, however, does provide some support to the notion that the complexes observed in Figure 8 are LPDII particles.

To our knowledge, this is the first report describing covalent stabilization of PEI/DNA polyplexes for subsequent inclusion in LPDII vectors. The concept of reversible cross-linking via reducible disulfide bonds has been investigated by other groups, but only in the context of

polyplex-mediated gene transfer (*26, 27*). The underlying theory behind this approach is that reversible disulfide cross-linking will confer stability to transfection complexes until intracellular reduction of the disulfide bonds takes place. If the transfection vectors are in fact able to gain access to the reducing environment of the cytoplasm, it is plausible that reduction of these bonds will occur, possibly through interaction with reduced glutathione. Accordingly, the data presented here indicate that the effects of cross-linking are reversible upon disulfide bond reduction with DTE in vitro. Furthermore, our results show that polyplexes are more stable in terms of dissociation in the presence of a competing polyanion after DSP cross-linking, and that inclusion of cross-linked polyplexes into LPDII vectors mediates variable transfection, depending on the lipid:DNA ratio and the presence of PEG-DSPE or folate-PEG-Chol. The combination of serum resistance conferred by the diolein/CHEMS liposomes and the increased stability owed to cross-linking may make LPDII vectors incorporating these features more stable in the systemic circulation after intravenous delivery. This improved stability may ultimately translate into higher gene expression and improved therapeutic effects. Based on these results, further evaluation of the transfection properties of covalently cross-linked LPDII vectors in an animal model is warranted.

ACKNOWLEDGMENT

In addition to a grant (RPG-99-097-01-MGO) from the American Cancer Society National Division, this work was partly supported by a development grant from the Parenteral Drug Association (PDA).

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BC025512C

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Characterization of a novel diolein-based LPDII vector for gene delivery

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Received 7 March 2002; accepted 18 June 2002

Abstract

LPDII vectors are non-viral vehicles for gene delivery comprised of polycation-condensed plasmid DNA (polyplexes) complexed with anionic pH-sensitive liposomes. Here, we describe a novel LPDII formulation containing polyethylenimine (PEI) polyplexes complexed with anionic pH-sensitive liposomes composed of diolein/cholesteryl hemisuccinate (CHEMS) (6:4 mol/mol). The pH-sensitivity of diolein/CHEMS liposomes was evaluated through quantitative fluorescence measurements of calcein release and particle size analysis. The results indicated that diolein/CHEMS liposomes are stable at physiological pH, but undergo rapid aggregation and fluorescence dequenching at pH values ≤5.0. Using a luciferase reporter gene, in vitro transfection of KB oral cancer cells showed that the transfection efficiency of LPDII vectors was superior to other well-characterized polyplexes and lipoplexes. Results further showed that gene delivery using diolein-containing LPDII vectors was dependent on the PEI nitrogen/DNA phosphate (N/P) ratio, the lipid/DNA weight ratio and the cell line being transfected. Replacing PEI with poly-L-lysine as the DNA condensing agent resulted in only a moderate reduction in transfection activity. Moreover, in contrast to LPDII formulations incorporating dioleoylphosphatidyl-ethanolamine (DOPE), the transfection efficiency of diolein-based LPDII vectors was sustained in media containing up to 50% fetal bovine serum. Since diolein-based LPDII vectors mediate efficient gene transfer and retain their transfection activity in the presence of serum, diolein may be a promising alternative to DOPE for the construction of non-viral vectors for in vivo gene delivery.

Keywords: Gene therapy; Non-viral vectors; pH-sensitive liposomes; Diolein; Polyethylenimine; LPDII

1. Introduction

Recently, significant efforts have been devoted to the development of safe and efficient vectors suitable for the systemic administration of gene therapy. Non-viral vectors, although generally not as efficient as viral vectors, are attractive because they are associated with fewer safety concerns and are easier to produce in clinically relevant quantities. In addition, they are potentially less immunogenic and have fewer restrictions on their capacity to carry DNA. Among non-viral vectors, lipoplexes are perhaps the best characterized [1,2]. Currently, however, their utility in gene therapy is hampered by: (i) toxicity

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0168-3659/02/$ – see front matter
PII: S0168-3659(02)00167-0

and immunostimulatory activities of the cationic lipids used for lipoplex assembly, (ii) limited in vivo transfection efficiency, and (iii) low tissue specificity. These shortcomings may be due to inadequate DNA condensation, poor colloidal stability and incompatibility with the abundance of negatively charged macromolecules present in the physiological environment [3–7].

In an attempt to overcome these obstacles, LPDII transfection vectors, consisting of polycation-condensed DNA entrapped in anionic pH-sensitive liposomes, have been developed [8]. To formulate LPDII vectors, polyplexes are first prepared by condensing plasmid DNA with a cationic polymer to form nanometric particles containing a net positive charge. These polyplexes are then complexed to anionic pH-sensitive liposomes, via electrostatic interactions, to form LPDII vectors with either a net positive or net negative charge [8]. The capacity to formulate neutral or negatively charged transfection vectors is important, since these vectors are potentially more compatible with the physiological environment of the systemic circulation. Furthermore, neutral or negatively charged vectors should be more amenable to tissue-specific delivery, after incorporation of a targeting ligand, by reducing non-specific electrostatic interactions with cells [8,9].

Poly-L-lysine (PLL) and polyethylenimine (PEI) have both been utilized in LPDII vectors as the DNA-condensing agent, with similar efficacy [10]. However, variability in the composition of the anionic liposomes used to make LPDII vectors drastically changes their transfection potential. For example, previously published results demonstrate that dioleoylphosphatidylethanolamine (DOPE)-based LPDII vectors mediate efficient gene transfer to cultured cells [8,10–14]. In contrast, when LPDII vectors are formulated with the non-fusogenic lipid dioleoylphosphatidylcholine (DOPC), a complete loss of transfection activity is observed [8].

Despite the success that has been attained using DOPE-containing LPDII vectors, their utility is currently limited by a loss of transfection efficiency in the presence of serum, thus presenting a major barrier toward their application as a systemic gene therapy vehicle. Therefore, in the present study, we describe the development and characterization of a novel 'serum-resistant' LPDII formulation, containing di-9-octadecenoylglycerol, or diolein, as the fusogenic lipid component. The pH-sensitive properties of the diolein/CHEMS liposomes were characterized, followed by an evaluation of the in vitro gene transfer properties of diolein-based LPDII vectors. Transfection efficiency was analyzed with respect to polyplex charge ratio, lipid/DNA weight ratio, serum concentration and cell lineage. The results indicate that incorporation of diolein into LPDII formulations could potentially enhance the usefulness of LPDII vectors as in vivo carriers for gene therapy.

2. Materials and methods

2.1. Materials

Poly-L-lysine (PLL, M_r~29,000), calcein, cholesteryl hemisuccinate (CHEMS), dimethyldioctadecylammonium bromide (DDAB), 1,2-dioleoyloxypropyl-3-trimethyl-ammonium chloride (DOTAP), and di-9-octadecenoyl-glycerol (diolein, ~85% 1,3- and 15% 1,2-isomer) were purchased from Sigma (St. Louis, MO). Polyethylenimine (PEI, M_r~25,000) was purchased from Aldrich (Milwaukee, WI). Dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL). Luciferase assay reagents were obtained from Promega (Madison, WI). Polycarbonate membranes and the handheld LiposoFast extruder were obtained from Avestin (Ottawa, ON). BCA protein assay reagents were purchased from Pierce (Rockford, IL). Tissue culture media and supplies were purchased from Life Technologies (Rockville, MD).

2.2. Plasmid DNA preparation

pCMV-Luc plasmid DNA, encoding the firefly luciferase reporter gene under control of the cytomegalovirus enhancer/promoter, was obtained as a gift from Dr. Leaf Huang at the University of Pittsburgh. Plasmid DNA was isolated and purified from DH5-α *E. coli* using the Qiagen mega plasmid purification kit (Qiagen, Santa Clarita, CA). DNA concentration and purity were quantified by UV absorbance at 260 nm and 280 nm on a Shimadzu

UV-160U Spectrophotometer. The structural integrity and topology of purified DNA was analyzed by agarose gel electrophoresis.

2.3. Preparation of calcein-containing liposomes

Calcein-loaded liposomes, composed of diolein/CHEMS (6:4 mol/mol) or DOPE/CHEMS (6:4 mol/mol), were prepared by a polycarbonate membrane extrusion method, as deserибed previously [15]. Briefly, a chloroform solution of the lipid mixture, containing 9 mg of total lipid, was dried as a thin film on the wall of a 13×100 mm glass tube under a stream of nitrogen gas. Residual chloroform was further removed by placing the tube in a desiccator under vacuum for 30 min. The lipid mixture was then hydrated with 1 ml of an 80 mM calcein solution prepared in phosphate-buffered saline (PBS: 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.5). The suspension was sonicated for 5 min in a bath sonicator (model 50HT, VWR Scientific), subjected to six cycles of freezing and thawing, and then extruded 10 times through a 0.2 μm pore-size polycarbonate membrane, using a handheld LiposoFast extruder. Unentrapped calcein was separated from liposomes by gel filtration on a 10-ml Sepharose CL-4B column equilibrated in PBS. The final calcein concentration in liposome preparations was calculated from the absorbance at 495 nm, using a molar extinction coefficient of 80,000 cm^{-1} M^{-1}.

2.4. pH sensitivity of liposomes

The release of liposome contents in response to decreasing pH was determined using a calcein dequenching assay, as described previously [16]. Fluorescence measurements were performed on a Perkin-Elmer LS-50-B spectrofluorometer operated with an FTWinlab (Morena Valley, CA) computer program. The excitation and emission wavelengths were set at 495 nm and 520 nm, respectively, with a slit width of 5 nm for both. Calcein-loaded liposomes, containing 45 μg of lipid, were added to 2 ml of PBS (pH 7.5) or sodium acetate buffers of variable pH (100 mM NaCl, 10 mM acetate, pH 5.0, 5.5, 6.0, and 6.5). After a 30-min incubation at 37 °C, calcein fluorescence was measured. Due to the effect of pH on the calcein absorption spectrum, the fluorescence intensities obtained at acidic pH were multiplied by a calibration factor. The percentage of calcein release was defined as:

$$\%\ \text{calcein release} = ((I_{pH} - I_o)/(I_{100} - I_o)) \times 100\%$$

where I_o is the fluorescence at neutral pH, I_{100} is the fluorescence after the addition of 0.15% Triton X-100 at neutral pH, and I_{pH} is the fluorescence intensity at acidic pH before the addition of Triton X-100.

The time-course of liposome aggregation in response to buffer pH was also determined over a pH range of 4.8–7.4. Liposomes, containing 60 μg of lipid, were added to 400 μl of buffers, adjusted to different pH, at room temperature or 37 °C. The mean particle diameter and size distribution of the liposomes were measured at various times by photon correlation spectroscopy on a NICOMP Submicron Particle Size Analyzer Model 370.

2.5. Preparation of LPDII vectors

Anionic liposomes, composed of diolein/CHEMS (6:4 mol/mol) or DOPE/CHEMS (6:4 mol/mol), were prepared by an ethanol injection method, as described previously [10]. Briefly, a chloroform solution of the lipid mixture, containing 3 mg of total lipid, was dried as a thin layer on the wall of a 13×100 mm glass tube under a stream of nitrogen gas. The lipids were then dried further in a desiccator under vacuum for 30 min. The lipid film was redissolved in 50 μl of ethanol, and was then quickly injected into 1 ml sterile HEPES buffer (20 mM, pH 8.0) under vortexing. The mean particle diameter for diolein and DOPE liposomes was found to be ~120 nm and ~100 nm, respectively. The liposome samples were stored at 4 °C, and were used for LPDII formation within 2 weeks of preparation.

To prepare LPDII particles, 1 μg plasmid DNA and the appropriate amount of polycation (PEI or PLL) needed to achieve a particular polycation nitrogen/DNA phosphate ratio were each diluted in 20 μl of serum-free RPMI 1640 growth media prior to mixing. After mixing, the solution was briefly vortexed, and the resulting polyplexes were incubated for 10 min at room temperature. Anionic liposomes, diluted in 40 μl of serum-free media,

were then added to the polyplexes under mild vortexing to form LPDII particles.

2.6. Preparation of polyplexes and lipoplexes

Cationic liposomes, with the composition DOTAP/DOPE (1:1 mol/mol) or DDAB/DOPE (1:1 mol/mol), were prepared as described previously [10]. To prepare lipoplexes, 1 μg plasmid DNA and various amounts of cationic liposomes, each diluted in 20 μl of serum-free RPMI 1640 growth media, were mixed under vortexing. Similarly, PEI/DNA polyplexes were prepared by mixing 1 μg plasmid DNA with the desired amount of PEI, each diluted in 20 μl of serum-free RPMI 1640 growth media, to generate a PEI nitrogen/DNA phosphate (N/P) ratio of 10 [17]. The resulting lipoplexes or polyplexes were incubated for 10 min at room temperature before use.

2.7. Cell culture and transfection procedures

KB human oral cancer cells were obtained as a gift from Dr. Philip Low at Purdue University (West Lafayette, IN). 24JK-FBP, a methylcholanthrene-induced mouse sarcoma cell line retrovirally transfected with the human folate receptor-α gene, was obtained as a gift from Dr. Patrick Hwu at the National Cancer Institute. Both cell lines were cultured in RPMI 1640 growth media supplemented with 10% fetal bovine serum (FBS). B16, a mouse melanoma cell line, F98, a human glioma cell line, and CHO, Chinese hamster ovary cells, were obtained as gifts from Dr. Rolf Barth at the Ohio State University Medical School. CHO cells were maintained in F12 nutrient media, while B16 and F98 cells were maintained in DMEM growth media, all supplemented with 10% FBS. Cells were cultured continuously as monolayers at 37 °C in a humidified atmosphere containing 5% CO_2.

For transfection studies, ~10^5 cells/well were seeded on 24-well plates 24 h prior to transfection, achieving 70–80% confluence. Unless otherwise specified, all transfection experiments were carried out in standard growth media containing 10% FBS, and were performed in triplicate. Cells were incubated with transfection vectors, containing 1 μg plasmid DNA, in 500 μl of culture media for 4 h at 37 °C. Alternatively, preliminary transfection studies were performed by incubating cells with transfection complexes for 2 h. The transfection media was then removed, and the cells were incubated for a further 24 h in fresh culture media before analysis of luciferase activity.

To assay luciferase gene expression, cells were rinsed three times with PBS prior to cell lysis (lysis buffer: 0.5% Triton X-100, 100 mM Tris–HCl, 2 mM EDTA, pH 7.8). The cellular lysates were then centrifuged at 13,000 rev./min for 2 min, and the supernatants analyzed for protein content, using the BCA protein determination assay, and luciferase activity, using a commercially available kit. Relative light units (RLU) were measured with a Mini-Lum luminometer (Bioscan, Washington, DC), and were converted to pg luciferase using a standard curve, generated under identical assay conditions, using recombinant luciferase standards.

3. Results

3.1. Determination of the optimal lipid ratio for diolein/CHEMS liposomes

To determine the optimal lipid ratio of diolein/CHEMS liposomes, preliminary stability and pH-sensitivity studies were performed with five liposome formulations prepared at a series of different molar ratios, namely 5:5, 6:4, 7:3, 8:2 and 9:1. Diolein/CHEMS liposomes prepared at molar ratios beyond 8:2 yielded large lipid aggregates, which persisted after extensive vortexing and sonication, and were, therefore, excluded from further evaluation. Preliminary stability results for the remaining three formulations, shown in Fig. 1A, indicated that diolein/CHEMS liposomes prepared at molar ratios of 5:5, 6:4 and 7:3 all exhibited sufficient colloidal stability at physiological pH, and similar sensitivity to acid-induced aggregation at a pH of 4.2. However, subsequent analysis of LPDII-mediated transfection activity in KB oral cancer cells, displayed in Fig. 1B, showed that LPDII vectors formulated with diolein/CHEMS liposomes, containing a PEI nitrogen/DNA phosphate (N/P) ratio of 4 and lipid/DNA (L/D, w/w) ratio of 5, prepared at molar ratios of 5:5 and 6:4 yielded >14-fold and 33-fold higher luciferase

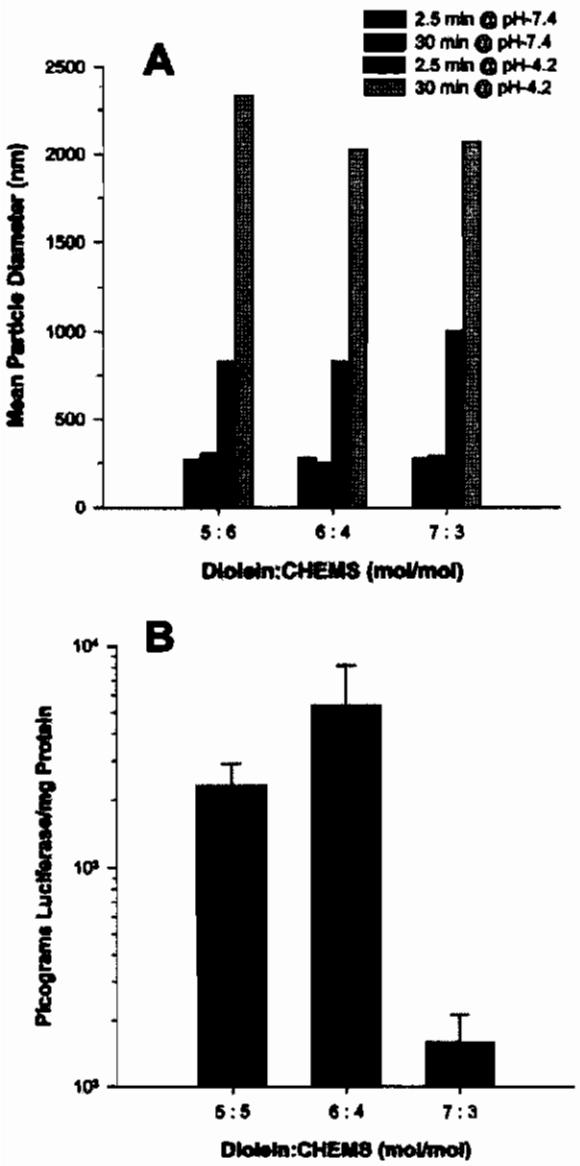


Fig. 1. Stability of diolein/CHEMS liposomes and transfection efficiency of diolein-based LPDII vectors using various diolein/CHEMS liposome formulations. (A) Mean diameter of diolein/CHEMS liposomes, prepared at molar ratios of 5:5, 6:4 and 7:3, evaluated over a 30-min time interval at pHs 7.4 and 4.2. (B) Transfection efficiency of LPDII vectors prepared at an N/P ratio of 4 and L/D ratio (w/w) of 5, containing diolein/CHEMS liposomes prepared at molar ratios of 5:5, 6:4 and 7:3. LPDII vectors contained 1 μg plasmid DNA, and transfections were carried out for 2 h on KB oral cancer cells in normal growth media containing 10% FBS. Data are presented as mean±S.D. ($n=3$).

activities, respectively, compared with LPDII vectors formulated with liposomes prepared at a molar ratio of 7:3. Furthermore, LPDII vectors incorporating liposomes prepared at a molar ratio of 6:4 yielded a luciferase activity >2-fold higher than that achieved with liposomes prepared at a molar ratio of 5:5. The 6:4 diolein/CHEMS lipid ratio was, therefore, selected for further characterization and incorporation into LPDII vectors in subsequent studies.

3.2. Acid-induced destabilization of diolein/CHEMS liposomes

Since the transfection activity of LPDII vectors is largely determined by the fusogenic properties of their lipid component, studies were performed to characterize the pH-sensitivity of diolein/CHEMS (6:4) liposomes using a calcein dequenching assay and partiele size analysis. Liposomes were prepared by polycarbonate membrane extrusion in the presence of 80 mM calcein, a concentration that generates complete self-quenching. An encapsulation efficiency of 2.6% was obtained, which is consistent with passive entrapment of calcein. The liposomes were then incubated in buffers of variable pH for 30 min at 37 °C, and analyzed by fluorometry for the degree of calcein dequenching. As shown in Fig. 2, the percentage of calcein released from diolein/CHEMS liposomes increased as the buffer pH decreased. At pH 5.0, 40% of the encapsulated calcein was released from diolein/CHEMS liposomes. In contrast, at the same pH, control liposomes composed of DOPE/CHEMS (6:4), a well-characterized pH-sensitive liposome formulation, released 67% of their contents.

To further assess the effects of pH on diolein/CHEMS liposomes, particle size analysis was performed to determine the extent and rate of liposome aggregation induced by acidic pH, both at RT and at 37 °C. The results, shown in Fig. 3A and B, reveal that liposome stability at physiological pH and aggregation at acidic pH are relatively independent of temperature, under the imposed experimental conditions. The mean diameter of diolein/CHEMS liposomes, after a 60-min incubation at a pH of 4.8, increased from ~150 nm to ~1700 nm and ~1500 nm at RT and 37 °C, respectively. In contrast, very little change in particle size was observed for

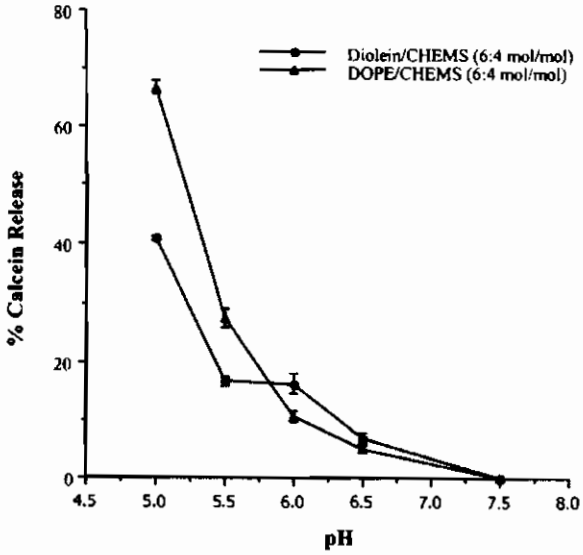
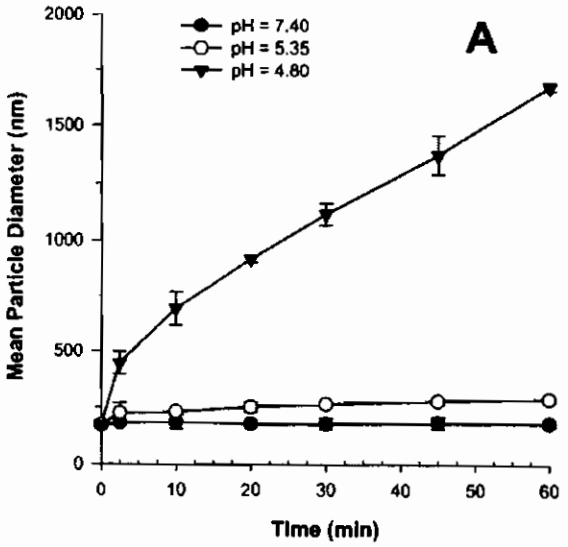


Fig. 2. Acid-induced calcein leakage from liposomes. Liposomes, composed of diolein/CHEMS (6:4 mol/mol) and DOPE/CHEMS (6:4 mol/mol), were incubated for 30 min at 37 °C in PBS (pH 7.5) or sodium acetate buffers of different pH. The fluorescence intensity was measured before and after addition of 0.15% Triton X-100. Percentage of calcein release was calculated as described in the Materials and methods. Data are presented as mean±S.D. ($n=3$).

liposomes incubated at pHs 7.4 and 5.35 for the same duration. Furthermore, at pH 4.8, particle size appeared to increase in a near linear fashion beyond a 10-min incubation period.

3.3. Transfection activity of diolein-containing LPDII vectors

To evaluate gene transfer efficiency, diolcin/CHEMS (6:4) liposomes were combined with PEI-condensed plasmid DNA, carrying the firefly luciferase reporter gene, to form LPDII vectors with a series of different L/D (w/w) and N/P ratios. The transfection activities of the various LPDII formulations were then evaluated in human KB cells, an oral carcinoma cell line. As shown in Fig. 4A, the transfection activity of LPDII vectors was dependent on both the N/P and L/D ratios. Luciferase gene expression initially increased with increasing L/D ratio, reaching a maximum between 1 and 2.5, before decreasing slowly with further increases in the L/D



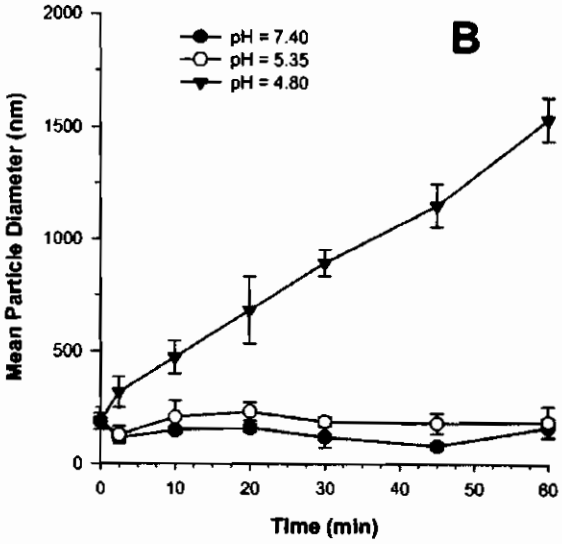
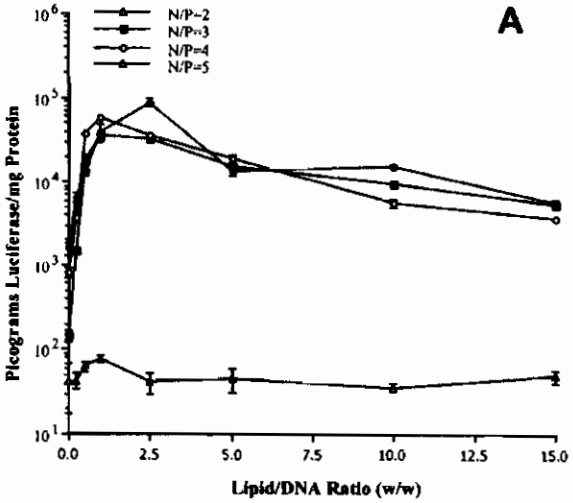


Fig. 3. Time-dependent effects of pH on the mean diameter of diolein/CHEMS liposomes. Diolein/CHEMS liposomes were incubated with buffers of various pH at (A) room temperature or (B) 37 °C, and liposome mean particle diameter was then measured at the indicated time points. Data are presented as mean±S.D. ($n=3$).



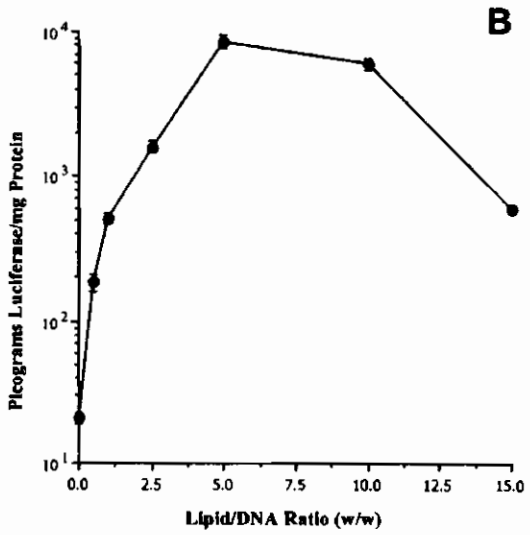


Fig. 4. Transfection of KB cells with LPDII vectors prepared with diolein/CHEMS liposomes. (A) Transfection efficiency of LPDII vectors prepared at various PEI nitrogen/DNA phosphate and lipid/DNA (L/D) ratios. (B) Transfection efficiency of LPDII vectors containing polylysine (N/P of 2) at various L/D ratios. LPDII vectors were incubated with KB cells in normal growth media containing 10% FBS for 4 h at 37 °C. The transfection medium was then replaced with fresh growth media, and luciferase activity was measured 24 h later. Data are presented as mean±S.D. ($n=3$).

ratio. Transfection activity was less dependent on polyplex N/P ratio, at ratios ≥3, although larger N/P ratios did appear to mediate higher luciferase activities at very low L/D ratios (≤0.25). At an N/P ratio of 2, where it has been shown that DNA condensation is incomplete, minimal transfection was observed, regardless of the L/D ratio.

To evaluate the role of the cationic polymer on transfection efficiency, LPDII vectors were also generated using poly-L-lysine (PLL) as the DNA-condensing agent. Because the ε-amino groups on lysine residues ($pK_a > 10$) carry a permanent charge at physiological pH, a PLL nitrogen/DNA phosphate ratio of 2 provided sufficient positive charge for DNA condensation and formation of LPDII vectors. Transfection of KB cells using PLL containing LPDII vectors showed only a modest reduction in reporter gene expression, compared to vectors incorporating PEI, as shown in Fig. 4B. Since PLL has been shown to have minimal endosomal lytic activity, the observed transfection activity using PLL-containing LPDII vectors is likely due to the fusogenic property of the diolein/CHEMS liposomes [18–20].

3.4. The effect of serum on LPDII transfection efficiency

As shown in Fig. 5, the presence of 10% FBS in the transfection media had a favorable effect on the transfection efficiency of diolein-containing LPDII vectors in KB cells, increasing luciferase gene expression by more than twofold compared to cells transfected in serum-free media. Significantly, further increases in the FBS concentration, up to 50%, did not result in a decrease in the transfection efficiency of diolein-containing LPDII vectors.

3.5. Comparison of the transfection activity among non-viral vectors

To assess the relative transfection efficiency of diolein-containing LPDII vectors, gene expression was compared against several non-viral formulations, including PEI polyplexes, two different lipoplex formulations and LPDII vectors prepared with DOPE/CHEMS liposomes (6:4 mol/mol). Lipoplex-

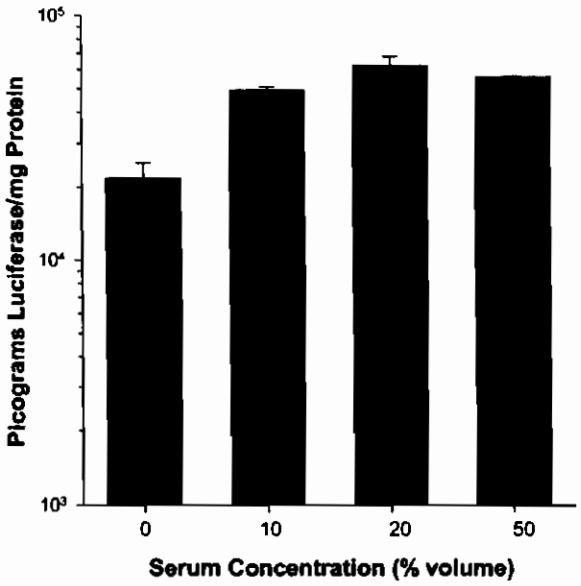


Fig. 5. Influence of serum concentration on the transfection activity of LPDII vectors prepared with diolein/CHEMS liposomes. The vectors were prepared at a PEI nitrogen/DNA phosphate ratio of 4, and a lipid/DNA weight ratio of 2.5. Transfection was carried out by incubating KB cells with transfection vectors for 4 h at 37 °C, in the presence of various concentrations of FBS (v/v). Data are presented as mean±S.D. ($n=3$).

es were prepared using two well-characterized cationic liposome formulations, namely DDAB/DOPE (1:1 mol/mol) and DOTAP/DOPE (1:1 mol/mol). As shown in Fig. 6, diolein-containing LPDII vectors produced >10-fold higher levels of luciferase activity compared to either lipoplex formulation. Similarly, PEI polyplexes, in the absence of pH-sensitive liposomes, also mediated lower transfection activity than diolein-containing LPDII vectors, suggesting that the lipid component in LPDII vectors promoted more efficient gene delivery. Furthermore, Fig. 6 also shows that transfection levels mediated by LPDII vectors formulated with DOPE/CHEMS liposomes were highly sensitive to the presence of serum, yielding luciferase levels orders of magnitude lower than those observed using DOPE-based LPDII vectors, in serum-free media, and for diolein-based LPDII vectors.

3.6. LPDII transfection efficiency in various cell lines

The transfection activity of LPDII vectors was further assessed in a number of different mammalian cell lines, including B16, a murine melanoma, 24JK, a sarcoma cell line, F98, a human glioma cell line, and CHO, Chinese hamster ovary cells. As observed with other non-viral vectors, the transfection efficiency of diolein-containing LPDII vectors was variable depending on the cell line being transfected, as shown in Fig. 7. With the exception of F98 cells, which exhibited luciferase expression of $\sim 1.5\times10^3$ pg luciferase/mg protein, transfection levels using diolein-based LPDII vectors were of the order of 10^4 pg luciferase/mg protein. Among the cell lines tested, KB cells consistently displayed the highest level of luciferase activity. Furthermore, based on visual inspection and quantitative measurements of protein concentration in cellular lysates after transfection, all cell lines appeared to tolerate LPDII-mediated transfection well.

4. Discussion

Successful implementation of gene therapy depends on the development of gene transfer vectors that are safe, efficient, and tissue specific. The theory behind the LPDII design is to increase transfection activity by combining the DNA condensing properties of cationic polymers with the endosomal lytic activity of fusogenic pH-sensitive liposomes. In fact, this design is reminiscent of the structure of an enveloped virus particle. Previous studies have shown that LPDII vectors mediate higher gene transfer efficiency and exhibit greater colloidal stability compared to some well-characterized lipoplexes and polyplexes [8]. Furthermore, LPDII vectors do not involve the use of cationic lipids, thus avoiding the potential toxicity resulting from non-specific stimulation of cytokine production [9].

Since endosomal escape of the DNA contained in LPDII vectors is likely mediated by the fusogenic properties of the lipid component, the anionic liposome composition has a profound impact on the transfection properties of LPDII vectors. All previ-

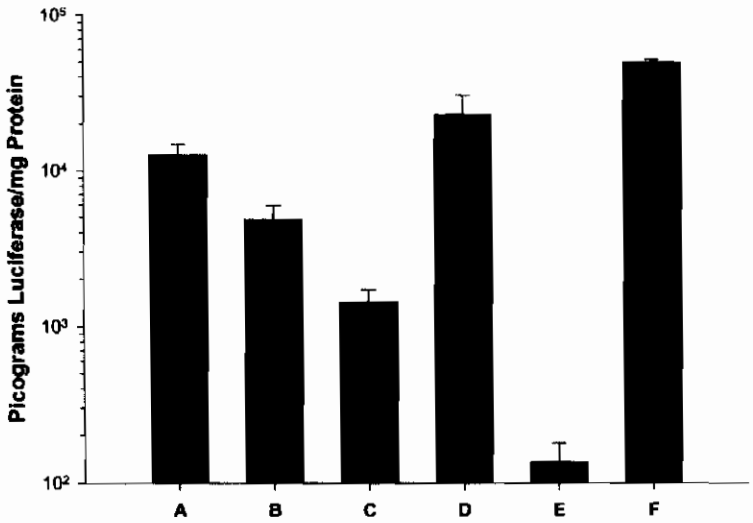


Fig. 6. Comparison of LPDII, PEI, and cationic liposome-mediated transfection. KB cells were transfected with 1 μg plasmid DNA prepared in various formulations. (A) PEI polyplexes, at a PEI nitrogen/DNA phosphate (N/P) ratio of 10. (B) DDAB/DOPE (1:1 mol/mol) lipoplexes, lipid/DNA (L/D) weight ratio of 5. (C) DOTAP/DOPE (1:1 mol/mol) lipoplexes, L/D weight ratio of 5. (D) LPDII vectors (composed of DOPE/CHEMS, 6:4 mol/mol) with an N/P ratio of 4 and an L/D ratio of 5, under serum-free conditions. (E) LPDII vectors (composed of DOPE/CHEMS, 6:4 mol/mol) with an N/P ratio of 4 and an L/D ratio of 5. (F) LPDII vectors (composed of diolein/CHEMS, 6:4 mol/mol) at an N/P ratio of 4 and an L/D ratio of 2.5. All transfections were carried out in the presence of 10% FBS, except where noted. Data are presented as mean±S.D. ($n=3$).

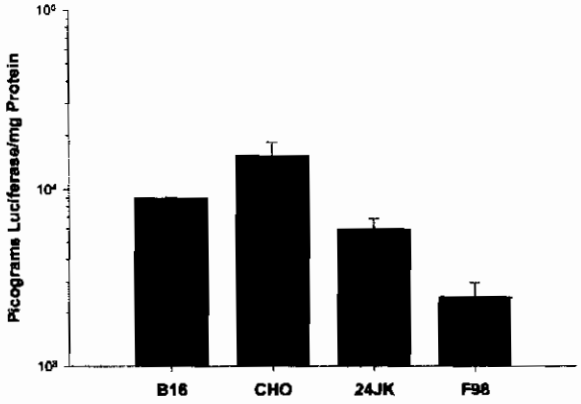


Fig. 7. Transfection efficiency of LPDII vectors in various cell lines. LPDII vectors were formulated with diolein/CHEMS liposomes and PEI-condensed DNA, prepared at an N/P ratio of 4 and a lipid/DNA weight ratio of 2.5. Data are presented as mean±S.D. ($n=3$).

ously reported, LPDII formulations have incorporated DOPE, or some derivation, as the pH-sensitive fusogenic lipid component [8,10–12]. DOPE is a cone-shaped molecule favoring membrane phase transition from a bilayer to a hexagonal II phase. In DOPE-containing liposomes, stability at neutral pH and pH-sensitivity are provided by the incorporation of an anionic amphiphile with a titratable headgroup, such as CHEMS, oleic acid, or citraconyl-DOPE [10–12]. The resulting liposomes are stable at neutral pH, but become fusogenic in response to the low pH found in endosomal compartments, thus providing a mechanism for endosomal disruption and cytosolic escape. A critical defect in pH-sensitive liposome formulations containing DOPE is that these liposomes tend to lose their fusogenic properties in the presence of serum, possibly due to modification of the bilayer composition by protein insertion and/or lipid extraction [21]. As a result, LPDII formulations containing DOPE are ineffective in the presence of serum, as shown in Fig. 6E, thus preventing

their utility for systemic gene delivery. Therefore, these studies were performed to characterize an alternative fusogenic lipid for LPDII assembly that remains active in the presence of serum, namely diolein.

Diolein, a diacylglycerol (DAG), has a cone-shape geometry similar to that of DOPE [22]. The polar portion of diolein, consisting of a single hydroxyl moiety, occupies a minimal hydrodynamic volume, which may be further reduced by its ability to form a hydrogen bond with the headgroup of an adjacent diolein molecule, as depicted in Fig. 8, or an oxygen atom on a nearby phospholipid molecule. These structural features predict a high propensity for diolein to promote the transition of lipids from a bilayer phase to an inverted hexagonal II phase, facilitating membrane fusion. In fact, it has been previously reported that diolein induces structural perturbations in phospholipid bilayers [23]. Moreover, the presence of diolein in pure phospholipid membranes has been shown to cause a marked decrease in the transition temperature from lamellar to hexagonal II phase [24,25]. Studies have also shown that treatment of liposomes with phospholipase C causes vesicle fusion, a process mediated by the formation of diolein [24,25]. In the present study, calcein-containing diolein/CHEMS liposomes showed pH-dependent calcein leakage, as well as

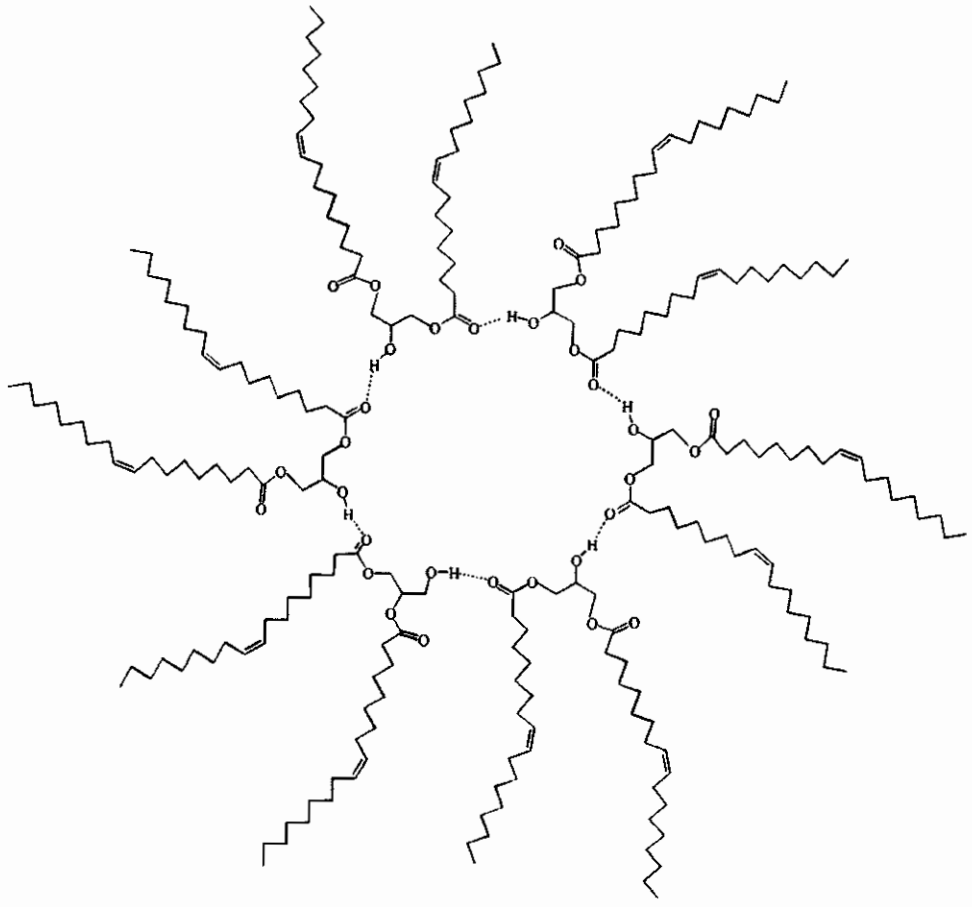


Fig. 8. Proposed structure of intermolecular hydrogen bonding between diolein molecules. Six molecules of diolein are drawn in the schematic diagram. Approximately 85% of the molecules are represented as the 1,3-isomer, with the remaining 15% represented as the 1,2-isomer, so as to reflect the composition of the diolein used in the liposome preparation.

irreversible particle size increase at low pH, similar to liposomes composed of DOPE/CHEMS. Although these results are consistent with membrane fusion in response to acidic pH, further studies are required to elucidate the mechanism of gene delivery mediated by diolein-containing LPDII vectors.

Branched PEI, with a molecular weight of 25 kDa, was used as the DNA condensing agent for LPDII formation in these studies. Based on published data, it is conceivable that PEI may improve gene delivery by facilitating nuclear localization of internalized DNA molecules following endosomal release [26]. However, the reported endosomal lytic activity of PEI is unlikely a main contributor to the transfection activity exhibited by the LPDII vectors characterized in this study. This is based on the fact that PLL, which has little endosomal lytic activity of its own [18–20], also produced significant luciferase activity when chosen as the DNA condensing agent for LPDII formation.

In contrast to LPDII particles prepared with reveal a gradual decline in transfection activity as the lipid/DNA ratio is increased, thus making this possibility somewhat less likely.

In light of the observed success using transfection vectors containing diolein, diolein may also find utility as a fusogenic lipidic component in other types of drug delivery vehicles, such as drug-carrying pH-sensitive liposomes or cationic liposomes. Furthermore, any safety concerns associated with the clinical use of diolein as gene/drug carriers should be minimal, since diolein is present endogenously in humans as a product of triglyceride metabolism [28]. In fact, ~1–15% of our dietary intake of oils and fats consists of diolein [29]. Moreover, diolein is a component of plant-based oils and fats, and is approved by the Food and Drug Administration for use as a component of emulsifiers [28].

5. Conclusion

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ANTICANCER RESEARCH 22: 1629-1634 (2002)

... Murine Lung Carcinoma Using Folate Receptor-targeted Liposomes

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... shown to selectively deliver entrapped agents into tumor cells via receptor mediated endocytosis. In this preliminary study, the biodistribution of FR-targeted liposomes were evaluated as a potential delivery agent for Na_3 ($B_{20}H_{17}NH_3$) for boron neutron capture therapy (BNCT) of FR(+) tumors. Materials and ... was incorporated into liposomes ... following which they were administered ... Results: show that received FR-targeted and non-targeted control liposomes showed ... levels of tumor boron uptake (up to 85 µg/g tumor) which reached a maximum at the 24 hour time-point, while the tumor-to-blood (T/B) ratio continued to rise until the ... Conclusion: High-level boron delivery is ... delivery agent. FR targeting does ... but may ... subcellular levels ...

... neutron capture therapy (BNCT) is being evaluated for the treatment of primary and metastatic brain tumors (1). The effectiveness of BNCT depends on the selective delivery of a sufficient concentration of the stable isotope, boron-10, ... cells, ... by irradiation with either thermal or ... The resulting nuclear capture and ... yield ... linear energy transfer ... liposomes have ... drug-carrying agents for neoplastic diseases. Due to their superior boron-carrying capacity, boronated liposomes have been evaluated as potential delivery agents for BNCT (2,3). Based on the enhanced permeability and retention (EPR) due to increased endothelial permeability and reduced lymphatic drainage, liposomes can passively accumulate in solid tumors. The tumor selective accumulation of boronated liposomes potentially can be further enhanced by introducing polyethyleneglycol (PEG)-derivatized lipids into the lipid bilayer to extend their systemic circulation time and a targeting ligand to increase selective tumor cell uptake (3).

Folate receptor (FR) is a glycosyl-phosphatidylinositol (GPI)-anchored membrane glycoprotein, which exhibits highly restricted normal tissue distribution and amplified expression in a variety of human cancers (4). Folic acid has been shown to retain its high affinity for FR upon derivation via its γ-carboxyl. Folate-derived bioconjugates, including liposomal drug carriers, have been successfully selectively targeted to FR-α in ovarian and other tumors, in which FR is overexpressed (5). Folate can be incorporated into liposomes via conjugation to either a phospholipid or a cholesterol anchor (6-9). In the present study, a highly-ionized boron rich agent Na_3 ($B_{20}H_{17}NH_3$) (Figure 1) was encapsulated into FR-targeted liposomes, which then were evaluated for their tumor localizing properties in an FR(+) murine tumor model.

Materials and Methods

Reagents. Na_3 ($B_{20}H_{17}NH_3$) (Figure 1), a trivalent anionic agent with high boron content (68% by weight), was kindly provided by Drs. M. Frederick Hawthorne and Kenneth Shelly from the Department of Chemistry of the University of California at Los Angeles, USA. Distearoylphosphatidylcholine (DSPC) and monomethoxy-polyethyleneglycol ... (2,000)-distearoylphosphatidylethanolamine ... were purchased from Avanti Polar Lipids (Alabaster, AL, ...

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Liposome preparation. FR targeted liposomes had the composition of DSPC/Chol/PEG-DSPE/f-PEG-Chol (65:30:3.5:0.5 mole/mole) while non-targeted control liposomes contained DSPC/Chol/PEG-DSPE (65:31.4, mole/mole), and were prepared by high-pressure polycarbonate membrane extrusion as follows. A mixture of lipids, containing 280 mg total phospholipids, was dissolved in chloroform, dried into a thin film under a stream of nitrogen, and then desiccated under vacuum for another 30 minutes. The dried lipids were hydrated in a 5 mL aqueous solution containing 152 mM $Na_3(B_{20}H_{17}NH_3)$. To facilitate lipid hydration, the mixture was subjected to vortex mixing followed by brief sonication in a bath sonicator and 6 cycles of freezing and thawing, which resulted in the formation of a heterogeneous population of lipidic vesicles. These were then extruded 6 times through a 100-nm pore-size polycarbonate membrane using a high pressure Lipex extruder with a 10 mL barrel heated to 60°C at 400 psi generated by compressed nitrogen gas. The resulting small unilamellar vesicles were purified from non-entrapped molecules by size exclusion chromatography on a Sepharose CL-4B column (Sigma Chemical Co. St Louis, MO, USA), pre-equilibrated in a buffer containing 10 mM sodium phosphate and 150 mM NaCl (phosphate-buffered saline, PBS, pH 7.4). The liposomes were eluted in the void volume fractions and the free boronated compound was eluted in later fractions. Boron concentrations in each of the fractions were quantified by means of direct current plasma-atomic emission spectroscopy (DCP-AES) using a Spectraspan VB spectrometer (Applied Research Laboratories, La Brea, CA, USA) as previously described (9). Encapsulation efficiency was determined by calculating the total boron content in the liposomal fractions and the total boron eluted from the column, including both the liposomal and the free boron fractions, as indicated in the following equation:

% encapsulation = (liposomal boron/(liposomal boron + free boron)) x 100%

The [illegible] ranged between [illegible] and 120 nm, and size distribution [illegible] by [illegible] spectroscopy [illegible] (NICOMP [illegible] submicron particle analyzer). Phospholipid [illegible] by an ammonium ferrothiocyanate [illegible] as previously described (10). [illegible] within 2 weeks of preparation [illegible] leakage (<[illegible]%) of the entrapped [illegible] by [illegible] fractionation on a Sepharose CL-4B column.

Cell culture and animal model studies. The M109 murine lung carcinoma cell line of BALB/c origin was obtained as a gift from Dr. Philip S. Low [illegible]. The cell line was chosen since it has been [illegible] high level [illegible] FR [illegible] as well as [illegible] for tumor growth in response to treatment with FR [illegible] agents (11). The cells were maintained in [illegible] with [illegible], penicillin [illegible], streptomycin, and 10% fetal bovine serum, which [illegible] was maintained as monolayers in a humidified [illegible] 5% CO_2 [illegible] 37°C.

Female BALB/c mice weighing 16-20 g, were purchased from Charles River Laboratories (Wilmington, Massachusetts USA) and were maintained on a folate-free rodent diet (Dyets Inc., Bethlehem, Pennsylvania, USA) for the duration of the study. Three days after arrival, the mice were implanted subcutaneously (s.c.) with M109 tumor cells. [illegible] a suitable size, they were excised, minced [illegible] and implanted s.c. into the left flank of BALB/c mice. [illegible] the tumors had reached an average size of [illegible] biodistribution studies were initiated [illegible] liposomes and free drug were administered [illegible] containing [illegible] of

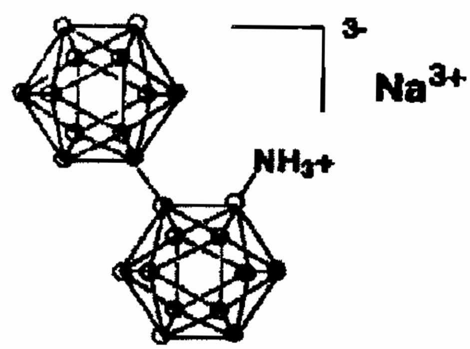

Figure 1. *Structure of $Na_3(B_{20}H_{17}NH_3)$.*

mice were euthanized by CO_2. Blood samples were obtained by cardiac puncture and the liver, spleen, kidney, and tumor were removed for tissue boron determinations.

Results

Liposome encapsulation of boron. Liposomal encapsulation of $Na_3(B_{20}H_{17}NH_3)$ was achieved by hydrating a lipid mixture in its solution followed by formation of unilamellar vesicles by repeated extrusion through a 100nm polycarbonate membrane using a high pressure Lipex extruder. The resulting encapsulation efficiency, as determined by size exclusion chromatography on a Sepharose CL-4B column, was ~ 15% for both FR-targeted and the non-targeted control liposomes. This value was consistent with a passive mechanism for the entrapment of the highly ionized agent into the liposomes.

Biodistribution of liposomal boron in tumor-bearing mice. BALB/c mice bearing s.c. syngeneic FR (+) M109 tumor were used to characterize the tumor localizing properties of liposome-encapsulated $Na_3(B_{20}H_{17}NH_3)$. Boron-containing liposomes or free drug were injected i.v. and tissue boron content was determined at 12, 24, 48, 72 and 96 hours following injection. Mice that received $Na_3(B_{20}H_{17}NH_3)$ had very low levels (<12 μg boron/g tissue) of boron in blood, liver, and kidneys at 12, 24 and 48 hour time-points, and undetectable levels at later time-points (data not shown), while in contrast, tumor boron concentrations were unmeasurable at all time points (data not shown). Biodistribution of FR-targeted liposomes and non-targeted control liposomes, as determined by tissue boron concentrations, are shown in Table I and Table II.

Table I. Boron Biodi[illegible]

Tissue

Blood
Liver
Spleen
Kidney
Muscle
Tumor
Skin
Tumor/Blood

[a]μg boron per gram
[b]A value of 0 indicat[illegible]

Table II. Boron Biod[illegible]

Tissue

Blood
Liver
Spleen
Kidney
Muscle
Tumor
Skin
Tumor/Blood

[a]μg boron per gram
[b]A value of 0 indica[illegible]

targeted control [illegible] highest levels (u[illegible] tissues. Very low [illegible] were found in the [illegible] boron were also [illegible] tumor-to-blood [illegible] increased over [illegible] received the FR [illegible]

...ion biodistribution of FR-targeted liposomes...

	[illegible]	24 hr	48 hr	72 hr	96 hr
	[illegible]	[illegible]	[illegible]	[illegible]	3.8 ± 3.2
	168.3 ± 5.6[a]	130.3 ± 24.2	172.0 ± 27.1	93.4 ± 16.8	58.5 ± 7.9
Spleen	156.7 ± 8.1	190[a] ± 3.9	134.3 ± 27.2	41.7 ± 13.4	32.1 ± 0.9
Kidney	39.0 ± 7.3	38.8 ± 5.6	26.0 ± 7.1	16.7 ± 2.5	10.5 ± 1.7
	3.7 ± 2.0	3.2 ± 1.7	0.0 ± 0.0[b]	0.0 ± 0.0	0.0 ± 0.0
	[illegible]	84.9 ± 47.6	31.5 ± 9.0	26.8 ± 10.7	21.0 ± 6.9
	2.8 ± [illegible]	[illegible]	5.1 ± 5.4	1.3 ± 2.3	0.0 ± 0.0
[illegible]	5.6 ± [illegible]	1.2 ± 0.7	2.4 ± 0.8	4.6 ± 2.2	5.9 ± 0.1

[a] Boron per gram weight tissue, presented as mean ± 1 standard deviation (n = 2-4).
[b] A value of 0 indicates that boron content was below the limited by detection by DCP-AES.

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	1.5 hr	24 hr	48 hr	72 hr	96 hr
Blood	140.4 ± 77.0[a]	72.3 ± 31.7	39.4 ± 10.0	7.3 ± 0.8	4.8 ± 1.5
Liver	172.1 ± 43.1	169.3 ± 92.4	157.5 ± 19.3	89.5 ± 18.6	48.6 ± 21.8
[illegible]	131.4 ± 11.2	186.8 ± 78.9	75.3 ± 13.5	29.6 ± 13.1	22.1 ± 6.1
[illegible]	38.1 ± 0.1	30.1 ± 4.1	21.4 ± 3.0	12.3 ± 3.3	8.9 ± 3.6
[illegible]	[illegible]	1.9 ± [illegible]	0.0 ± 0.0[b]	0.0 ± 0.0	0.0 ± 0.0
[illegible]	81.6 ± 26.7	64.6 ± 36.3	60.6 ± 15.1	31.0 ± 8.9	16.0 ± 6.8
[illegible]	2.6 ± 3.7	2.7 ± 2.3	6.2 ± 0.3	1.4 ± 2.4	0.0 ± 0.0
Tumor/Blood	0.5 ± 0.1	1.1 ± 0.8	1.2 ± 0.2	4.4 ± 1.8	3.3 ± 0.6

[a] Boron per gram weight tissue, presented as mean ± 1 standard deviation (n = 2-4).
[b] A value of 0 indicates that boron content was below the limited by detection by DCP-AES.

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...targeted control liposomes, the liver and spleen had the highest levels (up to 191 μg/g) of boron uptake among all tissues. Very low to undetectable levels of boron (< 4 μg/g) were found in the muscle. In contrast, relatively high levels of [illegible] were also found in the tumor (up to 85 μg/g). The tumor/blood ratios, compared to the non-targeted control liposomes (Tables I and II), The time-dependent changes to this ratio are shown in Figure 2.

Discussion

bearing FR(+) M 109 tumors. Mice that received the free drug did not show detectable levels of boron in the tumor, suggesting a lack of tumor selectivity of this agent. *In vivo* tumor localization of boronated liposomes potentially is facilitated by passive targeting due to increased endothelial permeability and limited lymphatic drainage frequently observed in solid tumors. In previous reports, tumor-targeted immunoliposomes and FR-targeted liposomes have shown a moderate enhancement in tumor accumulation compared to the non-targeted liposomes, but the degree of enhancement was highly variable. For example, transferrin receptor-targeted immunoliposomes showed only a 2-fold increase in boron localization in a rat xenograft model (13). Previous studies in cultured FR(+) KB human oral carcinoma cells have shown that FR targeted liposomal $Na_3(B_{20}H_{17}NH_3)$ was taken up via the FR, which was 49-fold more efficient in boron delivery than the non-targeted control liposomes, which could be blocked by co-incubation with 1 mM free folic acid (Pan *et al*, manuscript in revision). This study, however, showed indistinguishable tumor boron levels in mice that received FR-targeted liposomes and non-targeted control liposomes. These findings are consistent with a previous report in which biodistribution of FR-targeted liposomes was evaluated in an FR(+) 24JK-FBP murine sarcoma model (8). Higher liver and spleen uptake of FR-targeted liposomes could be attributed to increased uptake by the reticuloendothelial system (RES) due to incorporation of the lipophilic folate derivative folate-PEG-DSPE. The lower blood boron level for mice that received the FR-targeted liposomes may have resulted in the higher tumor/blood ratio at the 96-hour time-point compared to the non-targeted control liposomes.

Intratumoral and subcellular distribution of the boron are also important determinants for the effectiveness of neutron capture therapy. It is conceivable that FR-targeted liposomes may be more efficiently internalized by FR-bearing tumor cells following extravasation compared to non-targeted liposomes within the solid tumor. Furthermore, FR-mediated liposome targeting has been shown to overcome multi drug-resistance in tumor cells (12).

One of the most important questions relating to the use of FR-targeted liposomes for BNCT, is what histological tumor type might be potential targets? High-grade gliomas do not express amplified FR (Sun *et al*, unpublished data). Ovarian cancer cells express large numbers of FR, but the dissemination of this tumor within the abdominal cavity does not make it a suitable target for BNCT. However FR-targeted liposomes, when injected intraperitoneally, either alone or in combination with monoclonal antibodies directed against the FR, potentially might be useful in delivering cytoreductive chemotherapeutic agents to ovarian cancer cells. Melanoma cells can express amplified FR (Adams, unpublished data) and as this tumor spreads, the brain frequently becomes the site of metastases. Although FR-targeted liposomes would not be expected to

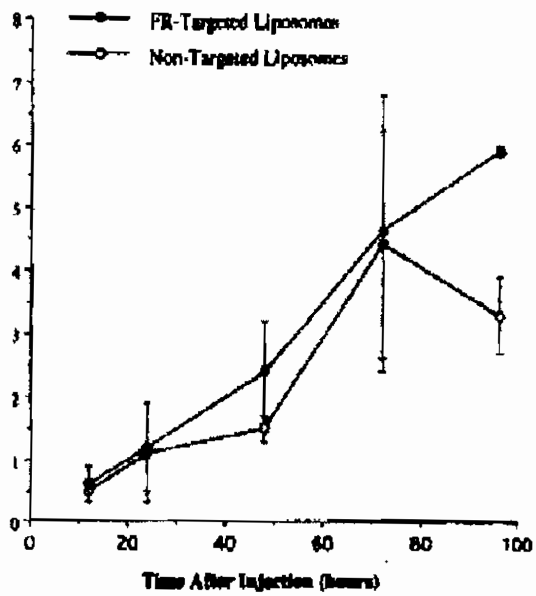

Figure 2. Time-dependent changes in tumor-to-blood ratio in tumor-bearing animals.

cross the blood-brain barrier due to their size (14), direct intracerebral infusion by means of convection-enhanced delivery (CED) might effectively disperse them (15). Since BNCT is a binary modality, one then could wait until the FR-targeted liposomes have cleared from normal brain and had preferentially concentrated in the metastatic nodules. The fact that choroid epithelial cells express amplified FR (16) should not present a problem since CED targets brain parenchyma and the FR-targeted liposomes would not be expected to enter the cerebrospinal fluid.

In summary, we have shown that liposomes can deliver up to 84 µg boron/g tumor to FR(+) M109 tumors *in vivo*, a level that exceeds that required (20-35 µg/g tumor) for sustaining a lethal $^{10}B(n,\alpha)^7Li$ capture reaction (1). Liposomal boron delivery to the tumor was much greater than to the muscle. Higher levels, however, were found in RES organs liver and spleen. Tumor localization of FR-targeted liposomes was indistinguishable from non-targeted control liposomes. Therefore, to demonstrate the potential benefit of FR targeting, future studies should focus on determining the intratumoral distribution and subcellular delivery of liposomes in tumors.

Acknowledgements

This work has been supported by the National Institute of Health grant ROICA79758 and Department of Energy grant DE-FG02-98ER62... USA.

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Received December 21, 2001
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% encapsulation =liposomal boron/(liposomal boron + free boron) × 100%

The mean diameter, which ranged between 110 and 120 nm, and size distribution of the liposomes were determined by photon correlation spectroscopy on a NICOMP 370 submicron particle analyzer. Phospholipid concentration was measured by an ammonium ferrothiocyanate partitioning colorimetric assay, as previously described (10). Liposome samples were stored at 4°C and used within 2 weeks of preparation, during which time no significant leakage (<1%) of the entrapped cargoes was detected by gel-filtration fractionation on a Sepharose CL-4B column.

Cell culture and animal model studies. The M 109 murine lung carcinoma cell line of BALB/c origin was obtained as a gift from Dr. Philip S. Low at Purdue University (West Lafayette, IN). This cell line was chosen since it has been previously exploited as an *in vivo* FR(+) model (11) as well as in an *in vivo* adoptive assay for tumor growth in response to treatment with FR-targeted agents (12). The cells were maintained in folate-free RPMI 1640 medium supplemented with 100 units/mL penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum, which was the only source of folate. The cells were cultured as monolayers in a humidified atmosphere containing 5%CO_2 at 37°C.

Female BALB/c mice, weighing 16-20 g, were purchased from Charles River Laboratories (Wilmington, Massachusetts, USA) and were maintained on a folate-free rodent diet (Dyets Inc., Bethlehem, Pennsylvania, USA) for the duration of the study. Three days after arrival, the mice were implanted subcutaneously (s.c.) with M109 tumor cell. Once the tumors had reached a sufficient size, they were excised, minced into 1-mm^3 cubes, and transplanted s.c. into the left flank of BALB/c mice. By three weeks, the tumors had reached an average size of ~ 300 mm^3, at which time *in vivo* biodistribution studies were initiated. Each experimental group consists of 3-5 mice. Liposomes and free drug were administered in ~ 200 μL aliquots containing 32 mg of boron per kg body weight (b.w.) by bolus intravenous (i.v.) injection via the lateral tail vein. At 12, 24, 48, 72 and 96 hour following injection, the mice were euthanized by CO_2. Blood samples were obtained by cardiac puncture and the liver, spleen, kidney, and tumor were removed for tissue boron determinations.

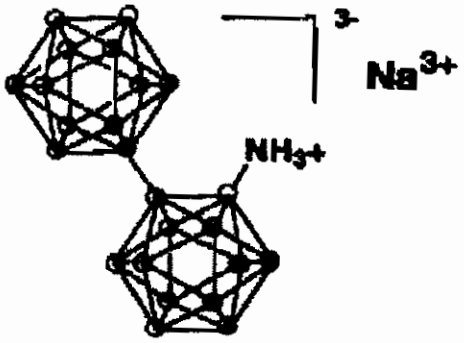

Figure 1. *Structure of Na_3 ($B_{20}H_{17}NH_3$).*

Results

Liposome encapsulation of boron. Liposomal encapsulation of Na_3 ($B_{20}H_{17}NH_3$) was achieved by hydrating a lipid mixture in its solution followed by formation of unilamellar vesicles by repeated extrusion through a 100nm polycarbonate membrane using a high pressure Lipex extruder. The resulting encapsulation efficiency, as determined by size exclusion chromatography on a Sepharose CL-4B column, was ~ 15% for both FR-targeted and the non-targeted control liposomes. This value was consistent with a passive mechanism for the entrapment of the highly ionized agent into the liposomes.

Biodistribution of Liposomal boron in tumor-bearing mice. BALB/c mice bearing s.c. syngeneic FR (+) M109 tumor were used to characterize the tumor localizing properties of liposome-encapsulated Na_3 ($B_{20}H_{17}NH_3$). Boron-containing liposomes or free drug were injected *i.v.* and tissue boron content was determined at 12, 24, 48, 72 and 96 hours following injection. Mice that received Na_3 ($B_{20}H_{17}NH_3$) had very low levels (<12 μg boron/g tissue) of boron in blood, liver, and kidneys at 12, 24 and 48 hour time-points, and undetectable levels at later time-points (data not shown), while in contrast, tumor boron concentrations were unmeasurable at all time points (data not shown). Biodistribution of FR-targeted liposomes and non-targeted control liposomes, as determined by tissue boron concentrations, are shown in Table I and Table II, respectively. For mice receiving either FR-targeted or non-

Table I. *Boron bioda*

Tissue
Blood
Liver
Spleen
Kidney
Muscle
Tumor
Skin
Tumor/Blood

[a]μg boron per gram
[b]A value of 0 indicat

Table II. *Boron biod*

Tissue
Blood
Liver
Spleen
Kidney
Muscle
Tumor
Skin
Tumor/Blood

[a]μg boron per gram
[b]A value of 0 indica

targeted control
highest levels (u
tissues. Very low
were found in th
boron were also
tumor-to-blood
increased over
received the FR
uptake in the li

Pharmaceutical Research, Vol. 19, No. 10, October 2002 (© 2002) **Research Paper**

Folate Receptor-Mediated Liposomal Delivery of a Lipophilic Boron Agent to Tumor Cells *in Vitro* for Neutron Capture Therapy

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Received June 1, 2002; accepted July 2, 2002

Purpose. This study was aimed at the *in vitro* evaluations of folate receptor (FR)-targeted liposomes as carriers for a lipophilic boron agent, K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$], in FR-overexpressing tumor cells for neutron capture therapy.

Methods. Large unilamellar vesicles (~200 nm in diameter) were prepared with the composition of egg PC/cholesterol/K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] (2:2:1 mol/mol), with an additional 0.5 mol % of folate-PEG-DSPE or PEG-DSPE added for the FR-targeted or nontargeted liposomal formulations, respectively.

Results. Boron-containing, FR-targeted liposomes readily bound to KB cells, an FR-overexpressing cell line, and were internalized via FR-mediated endocytosis. The boron uptake in cells treated with these liposomes was approximately 10 times greater compared with those treated with control liposomes. In contrast, FR-targeted and nontargeted liposomes showed no difference in boron delivery efficiency in F98 cells, which do not express the FR. The subcellular distribution of the boron compound in KB cells treated with the FR-targeted liposomes was investigated by cellular fractionation experiments, which showed that most of the boron compound was found in either the cytosol/endosomal or cell membrane fractions, indicating efficient internalization of the liposomal boron.

Conclusions. FR-targeted liposomes incorporating the lipophilic boron agent, K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$], into its bilayer were capable of specific receptor binding and receptor-mediated endocytosis in cultured KB cells. Such liposomes warrant further investigations for use in neutron capture therapy.

KEY WORDS: folate receptor; drug targeting; neutron capture therapy; boron; liposomes.

INTRODUCTION

Recent studies have reported amplified expression of the folate receptor (FR) in various types of human cancers, including breast, uterine, ovarian, and lung carcinomas (1). The receptor is generally absent in most normal tissues with the exception of choroid plexus, placenta, and low levels are expressed in lung, thyroid and kidney (2). The prevalence of FR overexpression in solid tumors, however, makes it a good marker for targeted drug delivery. Furthermore, FRs bind folic acid (an oxidized form of folate) with high affinity. For example, the FR α-isoform has a dissociation constant (K_d) for folic acid of ~0.1 nM, which is approximately 10-fold lower than its K_d for reduced folates (e.g., 5-methyltetrahydrofolate; 3). Furthermore, the FR has been shown to mediate the internalization of folate-derivatized liposomes into acidic endosomal compartments (4). Targeted delivery via the FR has been reviewed extensively (5,6). Incorporation into FR-targeted liposomes, therefore, could present a useful approach for the targeted delivery of a variety of therapeutic agents to tumor cells, including ^{10}B for neutron capture therapy.

Boron neutron capture therapy (BNCT), a binary cancer treatment, is based upon the capture reaction that occurs after irradiation of a stable ^{10}B isotope with thermal neutrons:

$$^{10}B + {}^{1}n \rightarrow {}^{7}Li + {}^{4}He\ (2.28\ MeV)$$

The resulting kinetic energy is distributed between the ionizing particles, ^4He (α particle), and $^7Li^+$ ions, which have effective ranges of <10 μm in tissue. Therefore, cellular damage is limited to those cells that have taken up the ^{10}B-containing agent and is equally lethal to oxic and hypoxic cells. However, relatively large intracellular accumulations of boron (approximately 20–30 μg of ^{10}B per gram of tumor) are necessary to produce cell death (7). BNCT requires the selective localization of boron within tumor cells to maximize damage to the tumor and minimize damage to surrounding normal tissue (8). The targeting of ^{10}B-containing agents to tumors has been reviewed recently (9). Such targeting strategies have included the use of drug carriers, such as liposomes (10–14, reviewed in 15), and low-density lipoproteins (16,17), as well as covalent conjugates of monoclonal antibodies (18–21) and epidermal growth factor (22–24).

Liposomal delivery of boron is an attractive approach for BNCT because liposomes are capable of carrying relatively large quantities of boron compounds. Low encapsulation efficiencies have resulted in the production of liposomes containing hydrophilic compounds (25). However, lipophilic boron compounds incorporated within the liposome bilayer result in an increase in the overall efficiency of incorporation of a ^{10}B-containing agent. Therefore, the gross boron content of the liposomes is increased in the formulation (13). In addition, liposomes show selective localization to tumors as a result of the increased extravasation and retention in tumor tissues as result of a porous endothelial lining and reduced lymphatic drainage. However, for an ideal ^{10}B-delivery system, liposomes should not only be able to selectively localize to the tumor tissue but also exhibit favorable cellular and subcellular distributions. Therefore, a cellular marker that would differentiate malignant from normal cells could be targeted to achieve selective delivery of boron compounds to tumor cells.

In this study, we have evaluated FR-targeted and nontargeted liposomal formulations that incorporate a lipophilic boron agent, K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] (13). Uptake studies were performed to determine the boron levels achievable with each of the liposomal formulations in FR (+) KB cells, using FR (−) F98 cells as a control. Furthermore, *in vitro* subcellular fractionation experiments were performed

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MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC), monomethoxy-polyethylene glycol-2000 (PEG-2000), and distearoylphosphatidylethanolamine (DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Calcein, cholesterol, folic acid dihydrate, Sepharose CL-4B resin, and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] was synthesized, as described previously (13). Tissue culture media, fetal bovine serum (FBS), and antibiotics were purchased from Life Technologies (Rockville, MD, USA). Folate-polyethyleneglycol-DSPE (f-PEG-DSPE) was synthesized as reported previously (26).

Cell Culture

KB cells, a human oral cancer cell line (ATCC# CCL-17) that overexpresses the FR, were given to us by Dr. Philip Low, Purdue University, West Lafayette, IN. KB cells were cultured as a monolayer in folate-free RPMI 1640 media supplemented with penicillin, streptomycin, and 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C. F98 glioma cells were cultured as a monolayer (ATCC# CRL-2397) in DMEM media supplemented with penicillin, streptomycin, and 10% FBS under the same conditions as those used for KB cells.

Preparation of Liposomes Incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]

FR-targeted and nontargeted liposomes were prepared using a previously described procedure (27) based on polycarbonate membrane extrusion. Briefly, egg PC, cholesterol, and K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] were dissolved at a 2:2:1 molar ratio in chloroform ($CHCl_3$). The FR-targeted and nontargeted liposome preparations contained an additional 0.5 mole % of f-PEG-DSPE or PEG-DSPE, respectively, which were added to the $CHCl_3$ solution and dried to a thin film in a round-bottom flask. The dried lipid was then hydrated in 1 mL of phosphate-buffered saline (PBS, 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) and dispersed by mixing. The resulting suspension of multilamellar vesicles were subjected to six cycles of freezing and thawing, then briefly sonicated, and extruded through a 0.2-μm pore size polycarbonate membrane using a handheld LiposoFast™ Extruder (Avestin Inc., Ottawa, ON, Canada). The resulting large unilamellar liposomes were separated from unincorporated K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] by gel-filtration on a Sepharose CL-4B column equilibrated with PBS. The mean diameter of the extruded liposomes (containing 35 mg of total lipid) was determined by photon correlation spectroscopy on a NICOMP Particle Sizer Model 370. The final boron concentration in the liposomes was determined by direct current plasma-absorption spectroscopy (DCP-AES), as previously described (28).

Boronated Liposomal Delivery in Cultured F98 Cells

F98 cells, an FR (–) cell line (data not shown), were washed with PBS and resuspended by treatment with 5 mM EDTA and then were further diluted in incubation media, pelleted by centrifugation at 400*g*, and resuspended in folate-free culture media at a density of 3×10^7 cells/mL. Various concentrations of FR-targeted or nontargeted liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] were added to the media and incubated at 37°C with gentle shaking for 2 h. After this, the media were removed by centrifugation at 400*g* for 5 min. To remove unbound, extracellular liposomes, the cells were washed three times with cold PBS by resuspension and centrifugation at 400*g*. The media, wash, and digested cell pellets were collected for boron analysis by DCP-AES.

Boronated Liposomal Delivery in FR-Bearing KB Cells

KB cells were washed with PBS and treated the same as above for the F98 cell-binding studies except that the cells were resuspended in folate-free culture media at a density of 1×10^7 cells/mL. For the competitive binding assays, 1 mM of free folate was added to the incubation media. Various concentrations of FR-targeted or nontargeted liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] were added to the KB cell suspension and incubated at 37°C with gentle shaking for 2 h. After incubation, the cell samples were prepared as described above for the F98 cell binding study.

To determine the intracellular distribution of cell-associated boron-containing liposomes, cells treated with FR-targeted and nontargeted liposomes encapsulating calcein were examined by fluorescence microscopy using a Zeiss Axioskop Epifluorescence Microscope with an Optonics three-chip, low-light level color CCD camera attachment. The cells were processed as described above except that they were treated with K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]-incorporated liposomes encapsulating 50 μM calcein. The cell pellets were then resuspended in 1 mL of PBS after incubation and cell washing. KB cell images were obtained in both the fluorescence (dark field) and phase-contrast (bright field) modes using a 40× objective. Digital images were collected and analyzed using the NIH Image 1.6 (Springfield, VA, USA) software.

KB cells that had been treated with K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]-incorporated liposomes encapsulating calcein were evaluated by fluorescence spectrophotometry. The cells were treated as described above except they were lysed with 0.1% (v/v) Triton X-100 after resuspension in PBS. All fluorescence measurements were performed using a Perkin-Elmer LS-5B spectrofluorometer operated with an FTWinlab (Morena Valley, CA, USA) software program. The excitation and emission wavelengths were set at 490 nm and 520 nm, respectively.

Intracellular Distribution of Boron in KB Cells

Subcellular fractionation experiments were performed using a previously described method (29). Briefly, KB cells

or nontargeted liposomes, incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$], were added to the media and were incubated at 37°C with gentle shaking for 2 h. After the incubation period, the media were removed by centrifugation at 400*g* for 5 min and the cells were washed three times with cold PBS. During the last wash, the cells were counted in a hemocytometer to determine cell viability and final cell concentration before fractionation. The cells were centrifuged at 400*g* for 3 min and then resuspended in 7 mL of cold distilled water and lysed by sonication on ice. The lysates were centrifuged at 1000*g* for 5 min and the pellet was resuspended in a 0.25 M sucrose/1.8 mM $CaCl_2$/1% Triton X-100 solution. A sucrose gradient was made by adding an equal volume of 0.34 M sucrose/0.18 mM $CaCl_2$ solution to the bottom of the tube, pushing up the lighter solution. The nuclear pellet was obtained after centrifugation at 600*g* for 10 min. The first supernatant was centrifuged at 3500*g* for 10 min to obtain the mitochondria pellet. The supernatant fraction was again centrifuged at 14,000*g* for 20 min to obtain the lysosomal pellet. Finally, the final supernatant contained the cytosol in addition to the endosomes. The samples were collected for boron analysis, by DCP AES.

RESULTS

Characterization of Liposomes Incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]

FR-targeted and nontargeted large unilamellar vesicles (~200 nm in diameter) incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] (7% w/w) into the liposomal bilayer were prepared and analyzed. As shown in Fig. 2, the FR-targeted and nontargeted liposomes showed ~100% incorporation efficiency, as evidenced by the absence of a free boron fraction. Furthermore, the liposomal formulations were stable when stored at 4°C in PBS for 8 weeks, as evidenced by an absence of boron leakage or change in particle size.

Cellular Uptake of Boronated Liposomes *in Vitro*

F98 and KB cells were incubated with FR-targeted and nontargeted liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] for 2 h at 37°C. To determine whether FR-mediated delivery was responsible for cellular uptake of boronated liposomes, FR (−) F98 cells were exposed to FR-targeted and nontargeted K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]-containing liposomes. As shown in Fig. 3, identical amounts (<0.2 μg of $^{10}B/10^7$ cells) of boron were delivered by FR-targeted and nontargeted liposomes to F98 cells.

To determine whether there was specific targeting to an FR (+) KB cell line, the cells were exposed to FR-targeted and nontargeted liposomes. Furthermore, a competitive binding assay was performed using 1 mM free folic acid. As shown in Fig. 4, uptake of the FR-targeted liposomes by KB cells was approximately 10 times higher than the nontargeted liposomes over the entire range of boron concentrations. In addition, the level of boron uptake achieved by FR-targeted liposomal delivery was reduced by 75% by coincubation with 1 mM free folate.

Comparison of the uptake efficiencies of the FR-targeted and nontargeted liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] taken up by either KB or F98 cells are shown in Fig. 5. The percent cellular uptake of boron was highest with the lowest boron concentration, and as concentration increased, the percent uptake decreased. Furthermore, the boron uptake in KB cells resulting from the delivery of FR-targeted liposomes was 35 to 64 times greater than that taken up by F98 cells, depending on the initial boron concentration, thereby establishing that uptake was receptor specific.

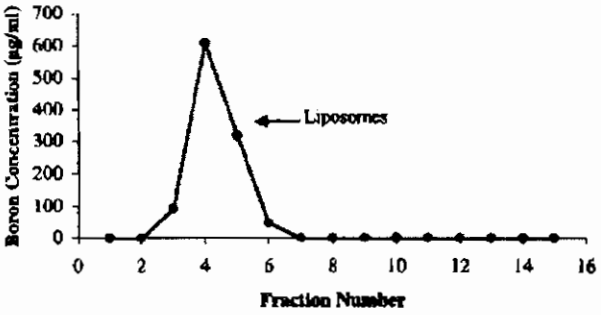
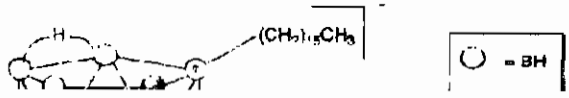
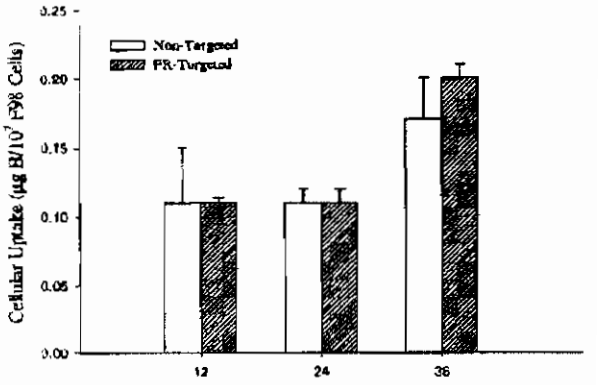


Fig. 2. Incorporation efficiency of the lipophilic boron agent into PEG-ylated folate receptor–targeted and nontargeted liposomes. Entrapment of the boron compound was determined by loading a 0.5-mL sample onto a 10-mL Sepharose CL-4B column. Fifteen consecutive 1-mL fractions were eluted with phosphate-buffered saline, and the amount of boron was determined by direct current plasma-absorption spectroscopy.





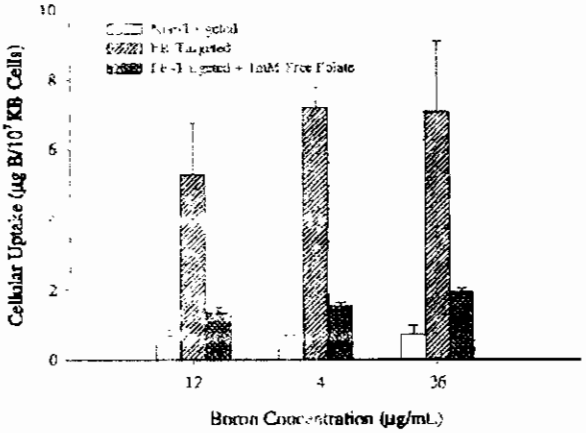


Fig. 4. Cellular uptake of boron delivered by folate receptor–targeted and nontargeted liposomes to cultured KB cells. A competitive binding assay was performed using 1 mM free folate. Cells were incubated with the boron-containing liposomes for 2 h at 37°C, as described in the Materials and Methods section. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

The intracellular distribution of cell-associated FR-targeted boronated liposomes (¹⁰B concentration = 24 μg/mL) encapsulating calcein was studied by fluorescence microscopy. As shown in Fig. 6, calcein fluorescence in KB cells exposed to FR-targeted liposomes was distributed throughout the cell, including the plasma membrane, intracellular vesicles, and cytosol. Cells treated with the FR-targeted liposomes in combination with 1 mM free folic acid exhibited much less fluorescence than FR-targeted liposomes, alone. However, the calcein fluorescence was distributed around the plasma membrane, as well as the cytosol/endosomal compartments as demonstrated by punctate fluorescence, which also was seen with the FR-targeted liposomes, alone. The cells exposed to nontargeted liposomes were shown to be associated with the cell surface, however, very little fluorescence was apparent.

The amount of calcein fluorescence was further quantified by fluorescence spectrophotometry. After the KB cells had been treated with 0.1% (v/v) Triton X-100, the fluorescence of the cell lysates were measured. The difference in calcein fluorescence between the FR-targeted and nontargeted liposomes (¹⁰B concentration = 24 μg/mL) associated with KB cells was approximately 10.5 times different, which was due to the differences in boron uptake at the same initial boron concentration.

Subcellular Distribution of K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] in Cultured KB Cells

The intracellular distribution of the boron compound was investigated by subcellular fractionation experiments. KB cells were incubated with FR-targeted and nontargeted liposomes (encapsulating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$]) for 2 h at 37°C with a boron concentration of 24 μg/mL because the greatest difference in boron uptake resulted at this concentration. Cell viability was greater than 90% after a 2-h incubation. The intracellular distribution of the boron delivered by either FR-targeted or nontargeted liposomes is shown in

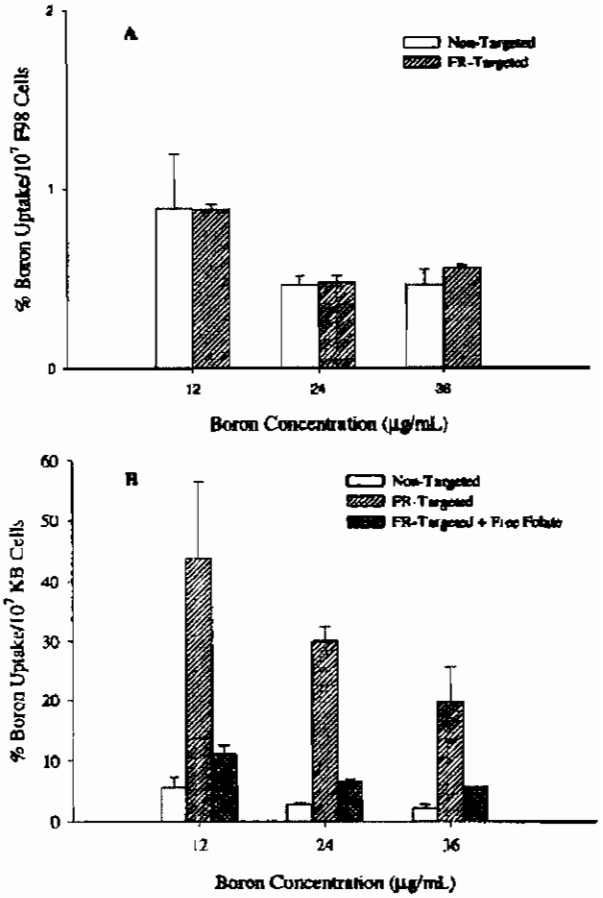
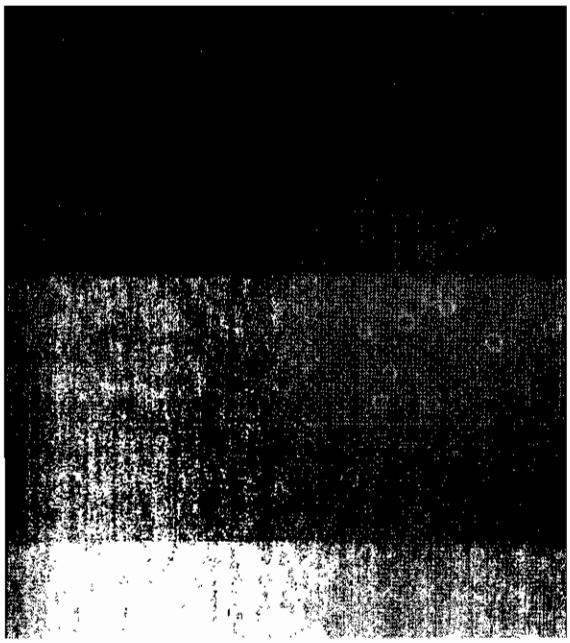


Fig. 5. The boron uptake efficiency by cultured (A) P98 cells and (B) KB cells after treatment with three different concentrations of boron-containing, folate receptor–targeted, or nontargeted liposomes. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

Fig. 7. Delivery of FR-targeted liposomes resulted in a total boron uptake of 587 μg $^{10}B/10^9$ KB cells. In addition, the cytosol/endosomes (~42% uptake) and the plasma membrane (~34% uptake) had the highest uptake of the various subcellular fractions. However, the lysosomal (~12% uptake), mitochondrial (~11% uptake), and nuclear (~1% uptake) fractions showed much reduced boron uptake in comparison to the other subcellular fractions.

The overall boron uptake resulting from the delivery by nontargeted liposomes was shown to be much lower (42.9 μg $^{10}B/10^9$ KB cells). The highest levels of boron were detected in the lysosomes (~33% uptake), mitochondria (~23% uptake), and on the plasma membrane (~22% uptake). In addition, boron delivered by nontargeted liposomes was undetectable in the cytosol/endosomes and the nuclear fractions. Overall, the boron uptake resulting from FR-targeted (587 μg $^{10}B/10^9$ KB cells) liposomal delivery exhibited much greater boron content in the subcellular fractions compared to nontargeted (42.9 μg $^{10}B/10^9$ KB cells) liposomes. These results demonstrate that FR-targeting specifically increased boron uptake by KB cells



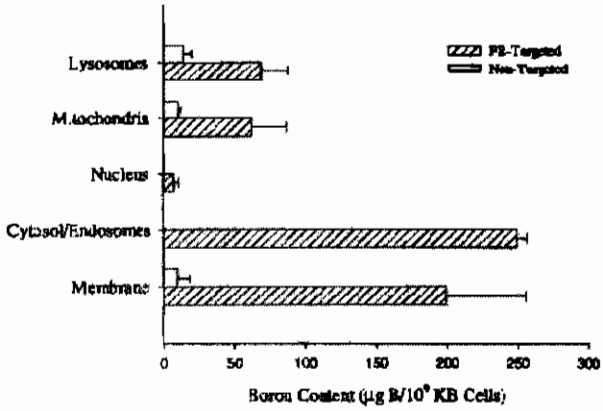


Fig. 7. Boron content of various subcellular fractions of KB cells measured by direct current plasma-absorption spectroscopy after exposure to either folate receptor–targeted or nontargeted, boron-containing liposomes for 2 h at 37°C, as described in the Materials and Methods section. Each data point represents the mean of at least three parallel experiments; error bars = 1 standard deviation.

Fl… re… ce… tar… μl… re… a r… pa… pa… fr… Lu… el…

DIS…

…R-targeted and non-targeted … incorporated the lipophil… This ind… in vitro … effi… the … boron-containing liposomes *in vitro* … distribution of boron in KB … cell line K[*nido*-7-CH… lipophilic boron agent that has been … incorporated into the liposomal … targeted … Furt… KB cells *in* … exposed … the lipo… n.L) for … The … showed remarkable intracellular … and … removed by extensive … the lipophilic properties of the com… upon uptake. K[*nido*-7-$CH_3(CH_2)_{15}$… embedded in the membranes

level of 20–30 μg ^{10}B/g of tissue would be sufficient for therapy, both FR-targeted and nontargeted liposomes would be able to provide enough boron for BNCT, provided that the compound was allowed to accumulate in the tumor for at least 2 h.

The potential *in vivo* use of FR-targeted liposomes for BNCT is dependent on the ability of these liposomes to selectively target FR (+) tumors. As shown in Fig. 3, the FR-targeted liposomes were not preferentially taken up by FR (–) F98 cells *in vitro*. However, FR-targeted liposomes exhibited over 10-fold greater boron uptake than the nontargeted type in KB cells *in vitro*, as evidenced by quantitation of boron and fluorescence microscopy. In addition, the free folate competitive binding assay resulted in a much lower boron uptake from the FR-targeted liposomes, indicating that the observed uptake was FR dependent. Furthermore, these results also were consistent with the fluorescence micrographs shown in Fig. 6. Whereas the nontargeted liposomes only showed minimal fluorescence around the plasma membrane, which was indicative of nonspecific cellular binding, the FR-targeted liposomes showed much greater membrane binding in addition to punctate intracellular fluorescence, which was indicative of FR-specific binding and endocytosis into intracellular vesicles.

An important factor in determining the effect of BNCT is the intracellular distribution of ^{10}B within tumor cells. The nucleus, and even more specifically the DNA, is the target of choice (30). A boron concentration, which is 2.5-fold higher than that localized in the cell nucleus and uniformly distributed within the target cell, however, has been shown to be as effective as targeting the nucleus (30). In our study, subcellular fractionation revealed that most of the intracellular boron delivered by FR-targeted liposomes was associated with either the plasma membrane or cytosol/endosomal fractions. The plasma membrane contained a large fraction of the over-

FR-mediated endocytosis and most likely were associated with the endosomal membranes. Furthermore, it has been shown that the subcellular distributions of boron and calcein, as detected by quantitative analysis and fluorescence microscopy, overlapped (Figs. 6 and 7). For example, the association of boron to the cell membrane resulting from the delivery of FR-targeted liposomes was demonstrated both by quantitative determination of boron and in the fluorescence micrographs, which showed increased fluorescence associated with the KB cell membranes. Furthermore, the resulting difference in boron uptake and fluorescence in KB cells between FR-targeted and nontargeted liposomes was the same (approximately 10-fold difference between FR targeted and nontargeted) at the same boron concentration.

For BNCT to be successful, it is necessary to obtain approximately 20–30 μg of ^{10}B per gram of tissue in the tumor cells. In this study, an f-PEG linker was used as a targeting ligand to direct boron-containing liposomes to FR-overexpressing cells. Folate, as a low molecular weight agent with high FR affinity, possesses several advantages as a targeting ligand. It is readily available and exhibits superior physiochemical stability with exposure to adverse storage conditions, organic solvents, and repeated freezing and thawing. Production with folate is consistent and relatively inexpensive since the necessary conjugation chemistry is well defined.

In conclusion, because folate is endocytosed, targeted moieties can accumulate intracellularly because of receptor recycling. In addition, because of a variety of FR (+) tumors, multiple tumor types could be targeted for neutron capture therapy. We have demonstrated that the introduction of FR targeting greatly increased the uptake of boron into KB cells. This suggests that strategies to further improve the intracellular boron uptake in malignant cells should focus on the use of liposomes, which are capable of carrying large quantities of ^{10}B-containing agents, in combination with FR-targeting. Further studies, including *in vivo* biodistribution, on the boron-containing FR-targeted liposomes are warranted to further assess the potential of these delivery vehicles.

ACKNOWLEDGMENTS

This work was supported by Grant R01CA79758 from the National Cancer Institute and the Department of Energy Grant DE-FG02-98ER6295.

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Biochimica et Biophysica Acta 1564 (2002) 31–37

BIOCHIMICA ET BIOPHYSICA ACTA
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A novel pH-sensitive liposome formulation containing oleyl alcohol

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Received 16 April 2001; received in revised form 7 March 2002; accepted 7 March 2002

Abstract

pH-sensitive liposomes are designed to undergo acid-triggered destabilization. First generation pH-sensitive liposomes, based on the cone-shaped lipid dioleoylphosphatidylethanolamine (DOPE), have been shown to lose fusogenicity in the presence of serum. Here, we report the design and evaluation of novel serum-resistant pH-sensitive liposome formulations that are based on the composition of egg phosphatidylcholine (PC), cholesteryl hemisuccinate (CHEMS), oleyl alcohol (OAlc), and Tween-80 (T-80). When loaded with the fluorescent probe calcein, these liposomes exhibited excellent stability at pH 7.4 and underwent rapid destabilization upon acidification as shown by calcein dequenching and particle size increase. Adjusting the mole percentages of T-80 and OAlc in the formulation could regulate the stability and pH-sensitive properties of these liposomes. Liposomes with a higher T-80 content exhibited greater stability but were less sensitive to acid-induced destabilization. Meanwhile, formulations with a higher OAlc content exhibited greater content release in response to low pH. The pH-triggered liposomal destabilization did not produce membrane fusion according to an octadecylrhodamine B chloride (R_{18}) lipid-mixing assay. Compared to DOPE-based pH-sensitive liposomes, the above formulations showed much better retention of their pH-sensitive properties in the presence of 10% serum. These liposomes were then evaluated for intracellular delivery of entrapped cytosine-β-D-arabinofuranoside (araC) in KB human oral cancer cells, which have elevated folate receptor (FR) expression. The FR, which is amplified in many types of human tumors, has been shown to mediate the internalization of folate-derivatized liposomes into an acidic intracellular compartment. FR-targeted OAlc-based pH-sensitive liposomes, entrapping 200 mM araC, showed ~17-times greater FR-dependent cytotoxicity in KB cells compared to araC delivered via FR-targeted non-pH-sensitive liposomes. These data indicated that pH-sensitive liposomes based on OAlc, combined with FR-mediated targeting, are promising delivery vehicles for membrane impermeable therapeutic agents.

Keywords: Folate receptor; Drug targeting; Cytosine arabinoside; pH-sensitive liposome; Drug delivery

1. Introduction

Liposomes are phospholipid bilayer vesicles that have been studied extensively as potential drug carriers. Major obstacles to liposomal drug delivery have been the slow drug release and lack of fusogenic activity of regular liposomes following internalization into the endosomal compartment. These factors contribute to the diminished efficiency of cytosolic delivery [1], especially when the encapsulated molecules are large or highly hydrophilic [2–4]. In order to overcome this obstacle, pH-sensitive liposomes have been developed that are stable at physiological pH but are destabilized upon acidification following cellular internalization, thereby, promoting the release of their contents into the cytosol [5–7].

Most reported formulations of pH-sensitive liposomes are composed of dioleoylphosphatidylethanolamine (DOPE), which has a strong propensity to form a nonbilayer structure due to its cone-shape geometry, and a weakly acidic amphiphile, such as cholesteryl hemisuccinate (CHEMS), which confers stability to the bilayer phase at neutral pH [8–12]. Under acidic conditions, CHEMS becomes partially protonated, thus losing its negative charge and, therefore, its ability to stabilize the bilayer structure, which is based on electrostatic repulsion. This further results in the destabilization and/or fusion of the liposomes. The DOPE-based pH-sensitive liposomes have been shown to improve the cyto-

liposomes as drug carriers *in vivo* is hampered by their relatively poor stability in the presence of serum [15–18]. Attempts to increase the serum stability of the DOPE-containing liposome formulations by incorporating a polyethyleneglyeol (PEG)-derivatized lipid resulted in significantly reduced pH-sensitivity [19].

The folate receptor (FR) is known to be overexpressed in a wide variety of solid tumors as well as in myeloid leukemias [20–22]. Folate conjugates of various therapeutic and diagnostic entities, including liposomes, have been shown to be internalized by cells via FR-mediated endocytosis into an acidic endosomal compartment [23–25]. In addition, folate-derivatized liposomes have been shown to selectively increase the delivery of cytotoxic drugs into tumor cells with elevated FR expression [26]. However, highly hydrophilic agents, such as cytosine-β-D-arabinofuranoside (araC), delivered by liposomes might be sequestered in the endosomal compartment resulting in a slow rate of cytosolic release. Combining FR-targeting, which promotes cellular endocytosis, with pH-sensitive liposomes, which promotes endosomal drug release, therefore, presents an attractive approach for improving the cytosolic delivery of araC.

In this study, we report the design and evaluation of novel pH-sensitive liposome formulations based on the use of oleyl alcohol (OAlc) in combination with egg phosphatidylcholine (PC) as the membrane destabilizing components. The stability and pH-sensitivity of the liposomes in buffer and in 10% serum were determined by fluorescence dequenching assays. Furthermore, the OAlc-containing liposomes, targeted to the FR, were evaluated for the intracellular delivery of araC. The possible mechanism for the pH-sensitivity of these liposomes is also discussed.

2. Materials and methods

2.1. Materials

Egg PC and DOPE were purchased from Avanti Polar Lipids (Alabaster, AL). Octadecylrhodamine B chloride (R_{18}) was purchased from Molecular Probes (Eugene, OR). Calcein, CHEMS, araC, folic acid dihydrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sepharose CL-4B resin, Tween-80 (T-80), and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). OAlc was purchased from Aldrich Chemical Co. (St. Louis, MO). Polycarbonate membranes and the handheld LiposoFast™ Extruder were obtained from Avestin Inc. (Ottawa, ON). Tissue culture media were purchased from Purdue University (West Lafayette, IN). The cells were cultured at 37 °C as a monolayer in folate-free RPMI 1640 media supplemented with antibiotics and 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2.

2.3. Liposome preparations

Liposomes encapsulating calcein were prepared using a procedure based on polycarbonate membrane extrusion, as described previously [27]. Briefly, a chloroform solution of the lipid mixture with the desired composition was dried into a thin film in a round-bottom flask on a rotary evaporator, and then further dried under vacuum. The lipid mixture was then hydrated in 80 mM calcein. The suspension was subjected to six cycles of freezing and thawing, briefly sonicated, and then extruded through a 0.1 μm pore-size polycarbonate membrane using a handheld LiposoFast™ Extruder. Unentrapped calcein was separated from the liposomes by gel filtration on a Sepharose CL-4B column equilibrated in phosphate-buffered saline (PBS, 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). The mean diameter of the extruded liposomes was determined by photon correlation spectroscopy on a NICOMP Particle Sizer Model 370. The final calcein concentrations in the liposome preparations were calculated based on absorbance at 495 nm using a molar extinction coefficient of 80,000 M^{-1} cm^{-1}.

2.4. Membrane fusion assay

The membrane fusion assay was carried out as described previously [28,29]. Briefly, labeled liposomes were prepared by incorporating a lipid soluble probe, R_{18}, at 5.7 mol% of the total lipid, a concentration that results in fluorescence self-quenching. Both labeled and unlabeled liposomes were prepared at a total lipid concentration of 2 mM. These were then mixed at a volume ratio of 1:4 (labeled/unlabeled). The liposome mixture was diluted to 5 μM lipids in the appropriate buffer, followed by a 10-min incubation at 37 °C. R_{18} fluorescence intensity was then measured at the excitation and emission wavelengths of 560 and 590 nm, respectively.

2.5. Fluorescence dequenching assay

Liposome content release was characterized using a calcein dequenching assay, as described previously [19]. All fluorescence measurements were performed using a Perkin-Elmer LS-5B spectrofluorometer operated with an FTWinlab (Morena Valley, CA) computer program. The excitation and emission wavelengths were set at 490 and 520 nm, respec-

100, which caused 100% calcein leakage from the liposomes. The percent of calcein release was calculated based on the equation:

$$\%\text{Calcein Release} = ((I_{pH} - I_o)/(I_{100} - I_o))100\%$$

where I_o was the fluorescence at pH 7.4, I_{pH} was the fluorescence intensity following incubation at acidic buffer pHs, and I_{100} was the fluorescence after the addition of Triton X-100. In addition, the changes in the mean particle size of the OAlc liposomes in an acidic environment were measured at preset time intervals.

2.6. Determination of liposome stability in the presence of serum

Liposomes entrapping 80 mM calcein and containing 40 nmol of lipid were added to 2 ml buffer at various pH values in the presence or absence of 10% newborn calf serum. After a 10-min incubation at 37 °C, calcein fluorescence was measured and percentage of calcein release calculated, as described above. In addition, liposome particle size measurements were also performed at various time points.

2.7. Cytotoxicity assay

The delivery of araC by OAlc liposomes was evaluated in KB cells. Preparation of araC-containing liposomes was carried out by the same method used for the preparation of calcein-containing liposomes described above, except 200 mM araC was used to hydrate the dried lipid mixture instead of calcein. In addition, 0.5 mol% of f-PEG-Chol was incorporated into the lipid mixture for the preparation of FR-targeted liposomes. The araC concentrations in the liposome preparations were calculated based on absorbance at 280 nm using a molar extinction coefficient of 475 M^{-1} cm^{-1}, after solubilizing the lipid membranes in a methanol/water (2:1) mixture. The encapsulation efficiency of these liposomes was found to be approximately 15%.

The cytotoxicity of various liposomal araC formulations was determined by the MTT assay, as described previously [30]. Briefly, KB cells were seeded in 96-well plates to reach ~ 25% confluence at the time of the study. The cells were treated in triplicate with 1:4 serial dilutions of the various araC formulations. Following a 2 h incubation at 37 °C, the cells were then washed with PBS. Fresh media, containing 10% fetal bovine serum, were then added and the cells were cultured for another 48 h. MTT was then added to the culture medium at a concentration of 0.6 mg/ml and the cells were incubated at 37 °C for an additional 2 h. The medium was then removed and the cells were dissolved in

3. Results

3.1. Formulation of pH-sensitive liposomes containing OAlc

All liposome compositions are described in molar ratios in this article. The pH-sensitive liposomes were designed using a primary lipid composition of egg PC/CHEMS (50:50) combined with varying amounts of T-80 and OAlc. T-80 was found be necessary since, in its absence, stable liposomes could not form at an OAlc to egg PC molar ratio above 1:2.

3.2. Effects of T-80 and OAlc content on acid-induced liposome destabilization

We predicted that the incorporation of T-80 would confer greater stability and reduced pH-sensitivity to egg PC-based liposomes, whereas increasing OAlc content would have the opposite effect. To evaluate this hypothesis, calcein-containing large unilamellar vesicles (LUV) (~ 100 nm in diameter) composed of egg PC/CHEMS/T-80/OAlc with two different T-80 contents and escalating OAlc-to-PC ratios were prepared and evaluated for pH-dependent calcein release.

First, liposomes with varying amounts of OAlc and a high T-80 content were compared for pH-dependent content release. As shown in Fig. 1A, liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:5:80) released 33% of their contents after a 10 min incubation at 37 °C at pH 5.0. In contrast, liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:5:20) showed only 6% calcein release under identical conditions. These findings suggested that elevated OAlc content resulted in increased pH-sensitivity of the liposomes.

Secondly, the above study was repeated using liposomes with a low T-80 content. Once again, the liposomes with higher OAlc content showed greater calcein release at acidic pH, as shown in Fig. 1B. Furthermore, the overall amount of calcein release was much higher compared to the above-described liposomes with a high T-80 content. For example, liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:2:80) showed 83% calcein release at pH 5. These findings suggested lowering T-80 content increased the pH-responsiveness of the liposomes.

Thirdly, we also examined non-pH-sensitive liposomes composed of PC/CHEMS/T-80 (50:50:2) using the same calcein dequenching assay. As anticipated, these liposomes showed only minimal calcein release (< 5%) at low buffer pH (data not shown). All liposome formulations evaluated in this study were also found to be stable (showing < 5% calcein leakage) over a period of 8 weeks in PBS (pH 7.4) when stored at 4 °C.

3.3. Effect of buffer pH on liposome particle size

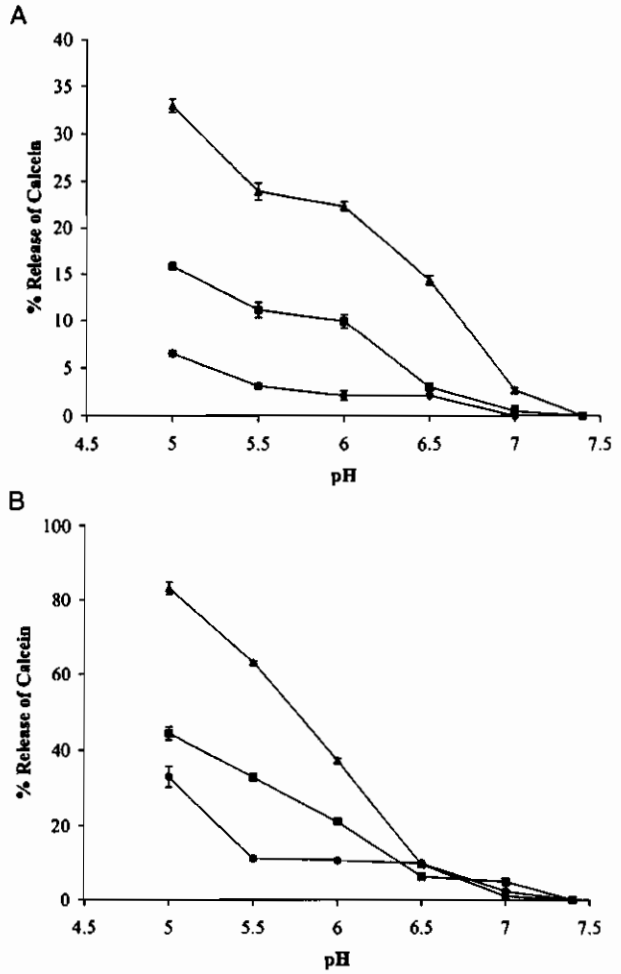

Fig. 1. Acid-induced calcein release from OAlc-containing liposomes with either high (Panel A) or low (Panel B) T-80 contents. Liposomes encapsulating 80 mM calcein were incubated for 10 min at 37 °C in buffers with a series of different pH values. Percent leakage of calcein was calculated based on fluorescenee measured before and after addition of Triton X-100, as described in Materials and methods. Each data point represents the mean of three parallel experiments; error bar = 1 S.D. The compositions of the liposomes used were egg PC/CHEMS/T-80/OAlc at molar ratios of: in Panel A, (●) 50:50:5:20; (■) 50:50:5:40; (▲) 50:50:5:80; and in Panel B, (●) 50:50:2:20; (■) 50:50:2:40; (▲) 50:50:2:80.

The increase in particle size was time dependent. No changes in particle sizes were observed when these liposomes were kept in pH 7.4 buffer.

3.4. Membrane fusion assay

To determine the likely mechanism of acid-induced liposomal destabilization, a membrane fusion assay based on lipid mixing was performed using liposomes containing a self-quenching concentration of the lipophilic probe R_{18}

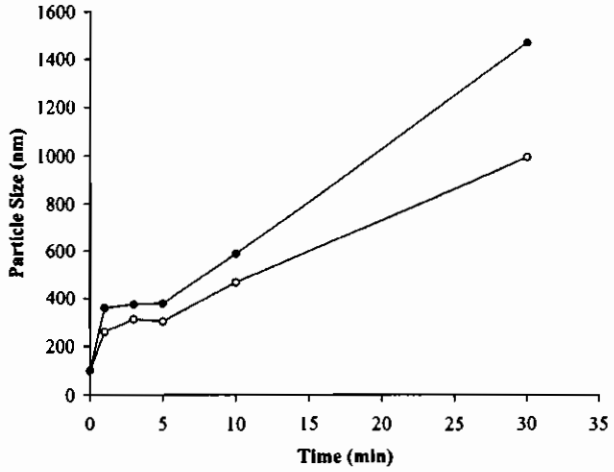

Fig. 2. pH-dependent particle size increase of OAlc liposomes incubated at pH 5.0 buffer. The liposomes were composed of egg PC/CHEMS/T-80/OAlc at molar ratios of (○) 50:50:5:80 or (●) 50:50:2:80.

OAlc (50:50:2:80). Meanwhile, liposomes composed of DOPE/CHEMS/R_{18} (60:40:5.7) and DOPE/CHEMS (60:40) were used as a positive control for the above assay. As shown in Fig. 3, the OAlc-containing liposomes did not show a significant increase in R_{18} fluorescence at any pH, suggesting a lack of membrane fusion. In contrast, R_{18} fluorescence from the DOPE/CHEMS liposomes gradually increased with decreasing pH, suggesting liposomal fusion. The degree of observed R_{18} fluorescence dequenching was

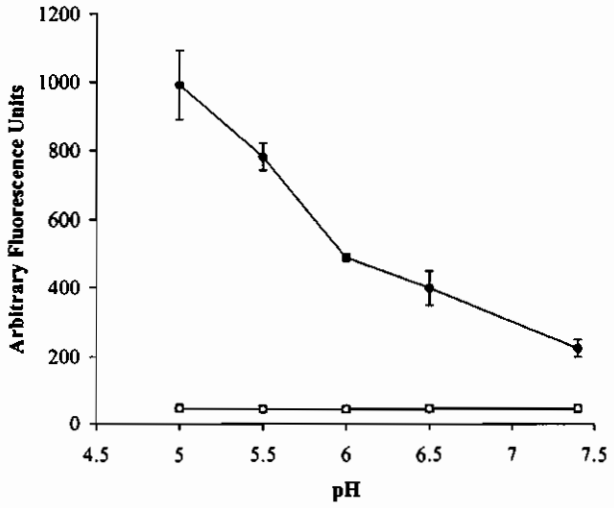

Fig. 3. Acid-induced R_{18} fluoreseence dequenching in pH-sensitive liposomes. Mixtures of labeled and unlabeled liposomes (at 1:4 ratio) were

much greater at pHs below 6, which correspond to the pK_a of CHEMS.

3.5. Stability and pH-sensitivity of OAlc liposomes in the presence of serum

For pH-sensitive liposomes to be utilized for *in vivo* drug delivery, they must retain both stability and pH-sensitive properties in the presence of serum. It has been previously reported that serum protein binding to liposomes negatively affect the acid-triggered destabilization of DOPE-based pH-sensitive liposomes [15–18]. We, therefore, examined the effect of serum on liposomes composed of egg PC/CHEMS/T-80/OAlc (50:50:2:80), using liposomes composed of DOPE/CHEMS (60:40) as a reference control formulation [31]. As shown in Fig. 4, the liposomes containing OAlc retained most of their pH-sensitive properties upon incubation with serum. Calcein release from these liposomes after a 10-min incubation at pH 5 was 56% in the presence of 10% serum. In contrast, liposomes composed of DOPE/CHEMS showed only 20% release under the same conditions. It was noted that for both OAlc and DOPE-based liposomes, serum exposure adversely affected the degree of calcein release in response to low buffer pH. The OAlc-based liposomes, however, were affected to a lesser degree, with a decrease of calcein leakage from 83% to 56%, compared to a decrease from 84% to 19% for the DOPE-based liposomes. Serum exposure at pH 7.4 did not significantly affect liposomal particle size over 24 h (data not shown).

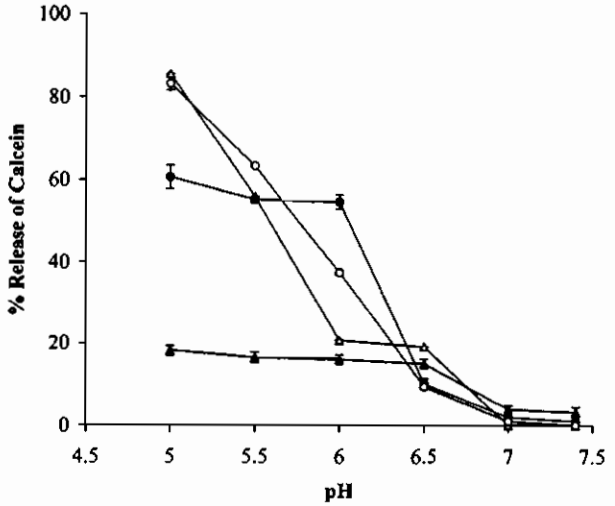

Fig. 4. Effect of serum on the pH sensitivity of OAlc liposomes. Liposomes composed of either egg PC/CHEMS/T-80/OAlc (50:50:2:80) or DOPE/CHEMS (60:40) were compared for pH-dependent calcein release in the presence or absence of 10% serum. Each data point represents the mean of three parallel experiments; error bar = 1 S.D. Conditions used: (▲) DOPE/CHEMS liposomes in the presence of 10% serum; (△) DOPE/CHEMS

Table 1
Cytotoxicity of various araC formulations in KB cells

Liposome formulation[a]	IC_{50} (μM)
FR-targeted, OAlc pH-sensitive liposomal araC	78
FR-targeted, OAlc pH-sensitive liposomal araC + 1 mM free folic acid	312
FR-targeted, non-pH-sensitive liposomal araC	1312
Non-targeted, non-pH-sensitive liposomal araC	> 10,000
Free araC + empty FR-targeted, OAlc liposomes	4375

[a] The liposome compositions were: FR-targeted, OAlc pH-sensitive liposomes: egg PC/CHEMS/T-80/OAlc/f-PEG-Chol (50:50:2:80:0.5); FR-targeted, non-pH-sensitive liposomes: egg PC/CHEMS/T-80/f-PEG-Chol (50:50:2:0.5); Nontargeted, non-pH-sensitive liposomes: egg PC/CHEMS/T-80 (50:50:2).

3.6. Delivery of araC to KB cells by FR-targeted OAlc-based pH-sensitive liposomes

To assess the potential utility of OAlc liposomes for increasing cytosolic drug delivery, cytotoxicity studies were carried out using FR-targeted liposomes entrapping araC in cultured KB cells. f-PEG-Chol, an FR-specific targeting ligand, was incorporated in the liposome formulation to facilitate FR-mediated cellular uptake. No significant change in the pH sensitivity of the targeted liposomes, as evaluated by the calcein dequenching assay, was observed with these liposomes (data not shown).

All araC-containing liposome formulations were found to be stable (< 5% leakage) over 6 weeks in PBS (pH 7.4) when stored at 4 °C. As shown in Table 1, FR-targeted pH-sensitive liposomal araC showed significantly elevated cytotoxicity, with an IC_{50} of 78 μM. When 1 mM free folic acid was present during drug incubation, the cytotoxicity was reduced by ~ 4-fold, with an IC_{50} of 312 μM, indicating that the observed araC delivery was FR-dependent. In contrast, FR-targeted non-pH-sensitive liposomal araC showed a much lower cytotoxicity towards KB cells (IC_{50} of 1312 μM). In the absence of receptor targeting, liposomal araC showed very low cytotoxicity (IC_{50}>10,000 μM), even when compared to the free drug. This was presumably due to the lack of a cellular uptake mechanism for the liposome entrapped drug. Empty pH-sensitive liposomes did not contribute significantly to cellular cytotoxicity (data not shown), as demonstrated by the resulting cytotoxicity of the free drug/empty liposome combination. These results indicated that both FR-targeting and a pH-sensitive liposomal composition were required for the efficient intracellular delivery of membrane-impermeable drugs, such as araC.

4. Discussion

In this study, we evaluated novel pH-sensitive liposome formulations that were based on the incorporation of a fatty alcohol, OAlc. This includes a detailed characterization of the pH-sensitivity and serum stability of these liposomes

lar delivery of the anticancer drug, araC, utilizing this novel liposomal formulation.

Previously reported formulations for pH-sensitive liposomes mostly incorporate the lipid DOPE as the principal component to promote bilayer destabilization. As shown in Fig. 4, these liposomes tend to lose most of their pH-sensitivity in the presence of 10% serum. Alternative compositions with greater resilience to serum are, therefore, needed for *in vivo* drug delivery. It has been suggested that OAlc, an unsaturated fatty alcohol, is capable of forming a hydrogen bond through its hydroxyl to an oxygen atom on the phosphate group on the PC molecule, resulting in the formation of a complex with geometry similar to that of DOPE, as illustrated in Fig. 5 [32]. This in turn could result in a lowering of the energy barrier for the lipid transition from a lamellar phase to a hexagonal II phase, which is required for membrane destabilization. However, a lipid mixing assay failed to show membrane fusion activity for the OAlc-containing liposomes in response to low buffer pH. Therefore, the observed pH-dependent leakage among these liposomes entrapping calcein might primarily be due to membrane destabilization, without indueing bilayer fusion.

Since the OAlc content in the lipid composition determines the extent of OAlc/PC complex formation, it should, therefore, be possible to increase the pH-sensitivity of the liposomes by increasing the OAlc content in the formulation. This concept has been demonstrated by the marked increase in calcein leakage when the OAlc content was increased in the liposomal formulation, as shown in Fig. 1.

The potential *in vivo* use of pH-sensitive liposome formulations is dependent on retention of their stability and pH-sensitivity in the presence of serum. As shown in Fig. 4, OAlc-containing liposomes demonstrated excellent stability and pH-sensitivity in the presence of serum. In addition, the degree of pH-sensitivity could be conveniently tuned by altering OAlc content in the formulation. Incorporation of T-80, which introduced a steric barrier on the surface of the liposomes, resulted in increased liposome stability while reducing its pH-dependent aggregation. Consequently, maintaining a delicate balance between these two factors is very important in creating an effective pH-sensitive liposomal formulation that is optimal for drug delivery.

AraC is a cytosine analogue widely used clinically as an anti-leukemic drug. AraC, which is highly hydrophilic, is

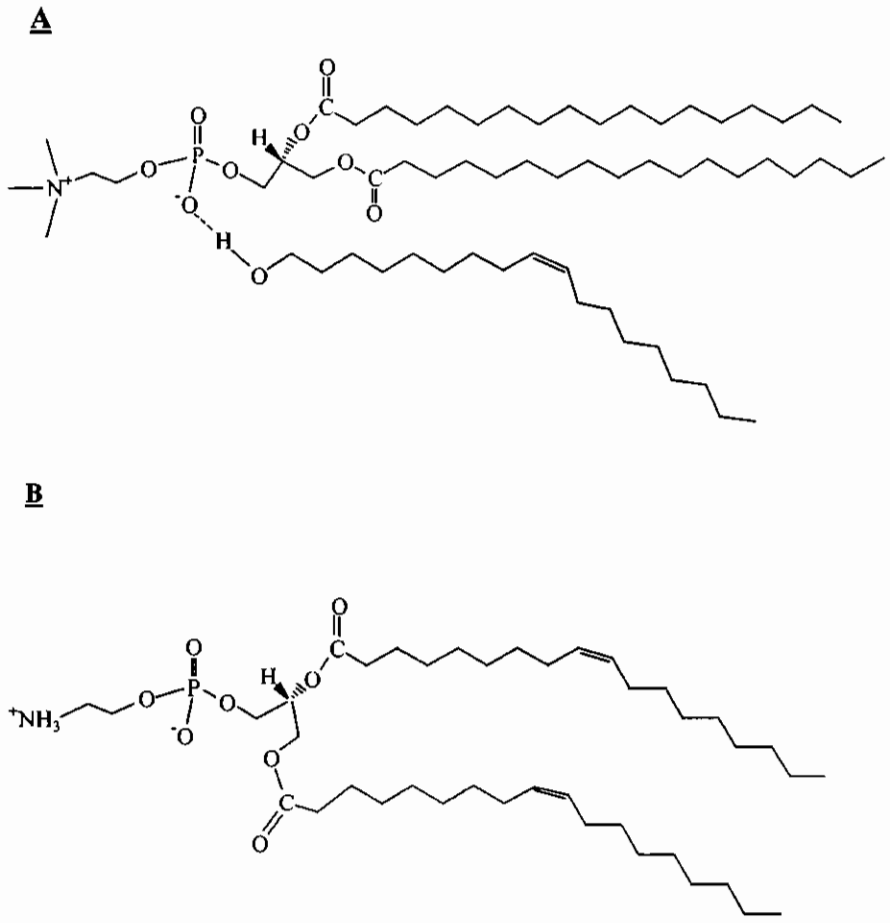

Fig. 5. Structures of (A) the proposed PC/OAlc complex and (B) DOPE. Note that, in both structures, the hydrophilic moieties occupy a relatively small volume compared to the lipophilic region. This should promote the formation of nonbilayer structures and liposome destabilization.

taken up by cells via a nucleoside transporter [33,34]. As a cell cycle-dependent agent, araC showed relatively poor cytotoxicity when added to cultured KB cells as a free drug (IC_{50} of 4375 μM) using an exposure time of 2 h. We, therefore, examined the effect of encapsulation of araC into FR-targeted liposomes, both pH-sensitive and non-pH-sensitive, on the *in vitro* cytotoxicity of this agent. As shown in Table 1, both FR-targeting and the use of a pH-sensitive formulation were found to be critical for maximizing liposomal araC cytotoxicity.

Since folic acid has high affinity for the FR and is stable, small, non-immunogenic, and readily available, it is ideal for exploration as a targeting ligand. Moreover, the FR is frequently overexpressed among human tumors, including ~ 70% of acute myeloid leukemias, while being absent in most normal tissues. FR-targeted liposomes can be readily produced in sufficient quantities for future clinical development. Therefore, FR-targeted OAlc liposomes, such as those entrapping araC, might have potential utility in the treatment of myeloid leukemias. FR-targeted delivery to solid tumors could also be possible given the high serum stability of the OAlc-containing liposomes, if prolonged systemic circulation and efficient endothelial extravasation could be achieved with the liposomes.

In addition to intracellular drug delivery, the pH-sensitive liposomal formulation described here may also constitute a drug delivery vehicle to pathological tissues, such as cancer, inflammation and infection sites, and ischemic areas, in which the pH is known to be lower than normal tissue [35]. Besides low molecular weight drugs such as araC, OAlc liposomes might find utility in the delivery of bioactive polypeptides, antisense oligodeoxyribonucleotides, as well as plasmid DNA in the delivery of gene therapy. Further studies on these liposomes, therefore, are warranted to explore these potential areas of application.

Acknowledgements

This work was partly supported by grant R01CA79758 from the National Cancer Institute and grant RPG-99-097-01-MGO from the American Cancer Society.

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Bioconjugate Chem. 2002, *13*, 435–442

Boron-Containing Folate Receptor-Targeted Liposomes as Potential Delivery Agents for Neutron Capture Therapy

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Received September 21, 2001; Revised Manuscript Received December 14, 2001

Boron neutron capture therapy (BNCT) depends on the selective delivery of a sufficient number of ^{10}B atoms to tumor cells to sustain a lethal $^{10}B(n,\alpha)^{7}Li$ reaction. Expression of FR frequently is amplified among human tumors. The goal of the present study was to investigate folate receptor (FR)-targeted liposomes as potential carriers for a series of boron-containing agents. Two highly ionized boron compounds, $Na_2[B_{12}H_{11}SH]$ and $Na_3(B_{20}H_{17}NH_3)$, were incorporated into liposomes by passive loading with encapsulation efficiencies of 6% and 15%, respectively. In addition, five weakly basic boronated polyamines were investigated. Two were the spermidine derivatives: N^5-(4-carboranylbutyl)spermidine·3HCl (SPD-5), N^5-[4-(2-aminoethyl-*o*-carboranyl)butyl]spermidine·4HCl (ASPD-5). Three were the spermine derivatives: N^5-(4-carboranylbutyl)spermine·4HCl (SPM-5), N^5-[4-(2-aminoethyl-*o*-carboranyl)-butyl]spermine·5HCl (ASPM-5), and N^5,N^{10}-bis(4-carboranylbutyl)spermine·4 HCl (SPM-5,10). These were incorporated into liposomes by a pH-gradient-driven remote-loading method with varying loading efficiencies, which were influenced by the specific trapping agent and the structure of the boron compound. Greater loading efficiencies were obtained with lower molecular weight boron derivatives, using ammonium sulfate as the trapping agent, compared to those obtained with sodium citrate. The in vitro uptake of folate-derivatized, boronated liposomes was investigated using human KB squamous epithelial cancer cells, which have amplified FR expression. Higher cellular boron uptake (up to 1584 μg per 10^9 cells) was observed with FR-targeted liposomes than with nontargeted control liposomes (up to 154 μg per 10^9 cells), irrespective of the chemical form of the boron and the method used for liposomal preparation. KB cell binding of the FR-targeted liposomes was saturable and could be blocked by 1 mM free folic acid. Our findings suggest that further evaluation of FR-targeted liposomes is warranted to assess their potential as boron carriers for neutron capture therapy.

INTRODUCTION

Boron neutron capture therapy (BNCT) currently is being evaluated as a modality for the treatment of primary and metastatic brain tumors and cutaneous and metastatic melanoma (Soloway et al., 1997; Barth et al., 1999; Hawthorne, 1998; Mishima et al., 2001). The effectiveness of BNCT depends on the selective accumulation of a sufficient concentration of the stable isotope, ^{10}B, in cancer cells, followed by neutron irradiation. The resulting nuclear reaction ($^{10}B[n,\alpha]^{7}Li$) yields tumoricidal high linear energy transfer (LET) α particles and recoiling ^{7}Li nuclei. Our efforts to improve the efficacy of BNCT have concentrated on (i) the development of novel boronated agents that have high boron content and exhibit selective uptake by tumor cells and favorable subcellular distribution (Soloway et al., 1997; Capala et al., 1996 and 1997) and (ii) methods to optimize the delivery of the two drugs that currently are being used clinically for BNCT: sodium borocaptate ($Na_2B_{12}H_{11}$-SH or BSH) and boronophenylalanine (BPA) (Barth et al., 1997 and 2000; Yang et al., 1997 and 2000). To maximize boron content, BNCT agents often consist of derivatives of one or more carborane cages, which contain 10 boron atoms. Boronated polyamines are potential delivery agents for BNCT due to their DNA-binding properties (Zhuo et al., 1999; Cai et al., 1997). The structures of a series of such derivatives, including two spermidine derivatives SPD-5 and ASPD-5, and three spermine derivatives SPM-5, ASPM-5, and SPM-5,10 (with two carborane cages), are shown in Figure 1. The polyamine moiety and, when present, the extra amino group provide hydrophilicity and aqueous solubility to these constructs. In contrast, the carboranyl moieties provide high molecular boron content, lipophilicity, and potential bilayer permeability upon deprotonation of the amino groups. Recently, a series of DNA-binding carborane-derivatized polyamines have been synthesized to facilitate the targeting of cellular DNA (Cai et al., 1997; Zhuo et al., 1999). Due to their superior boron-carrying capacity, Hawthrone and Shelly (1997) have carried out extensive studies on the incorporation of a variety of boronated agents into liposomes. The tumor-selective accumulation of boronated liposomes potentially can be further enhanced by introducing poly(ethylene glycol) (PEG)-derivatized lipids into the lipid bilayer to extend their systemic circulation time and a targeting ligand to increase selective tumor cell uptake (Yanagie et al., 2000).

FR, also known as the folate binding protein, is a glycosyl-phosphatidylinositol (GPI)-anchored membrane glycoprotein, which exhibits highly restricted normal

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10.1021/bc015557y CCC: $22.00
Published on Web 03/15/2002

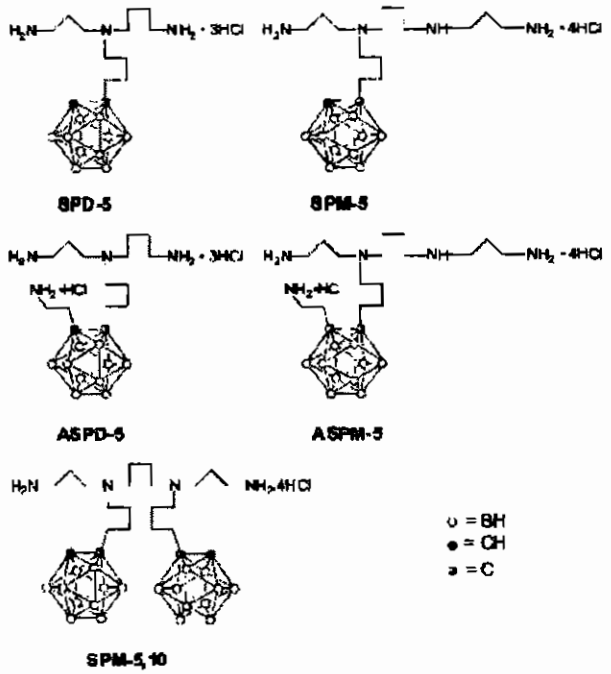

Figure 1. Structures of boronated polyamine derivatives that were studied.

tissue distribution, and is amplified in a variety of human cancers (Antony, 1996). Folic acid has been shown to retain its high affinity ($K_a \sim 10^{10}$ M^{-1}) for FR upon derivatization via its γ-carboxyl (Leamon and Low, 1991). Folate-derivatized bioconjugates, including liposomal drug carriers, have been successfully selectively targeted to FR-α in ovarian and other cancers, in which FR is overexpressed (Sudimack and Lee, 2000; Reddy and Low, 1998). Folate can be incorporated into liposomes via conjugation to either a phospholipid or a cholesterol anchor (Lee and Low, 1994; Gabizon et al., 1999, Guo et al., 2000). Passive and remote loading (Muggia, 2001) have been used to incorporate hydrophilic molecules into liposomes. The former is based on producing the liposomes in an aqueous solution of the molecule of interest, thereby encapsulating it in the entrapped volume. Typically, this method has limited encapsulation efficiency. In contrast, the remote loading method relies on the establishment of a transbilayer pH gradient in preformed liposomes, which contain a "trapping" agent consisting of a counterion (Nichols and Deamer, 1976; Johnsson et al., 1999) and often yields near-quantitative drug loading. Remote loading, however, is only applicable to weakly basic molecules that exhibit membrane permeability at a slightly basic pH, e.g., anthracyclines doxorubicin and daunorubicin (Muggia, 2001).

In the present study a series of boron containing polyamine derivatives have been incorporated into liposomes targeted to the tumor cells via the folate receptor (FR). Following this, we have evaluated the liposomal loading efficiency and in vitro tumor cell binding properties of a series of boronated compounds, including the anionic agents BSH and $Na_3(B_{20}H_{17}NH_3)$ (Feakes et al., 1994; Shelly et al., 1992) and cationic boronated polyamines. Two were the spermidine derivatives N^5-(4-carboranylbutyl)spermidine·3HCl (SPD-5) and N^5-[4-(2-aminoethyl-*o*-carboranyl)butyl]spermidine·4HCl (ASPD-5). Three were the spermine derivatives N^5-(4-carboranylbutyl)spermine·4HCl (SPM-5), N^5-[4-(2-aminoethyl-*o*-carboranyl)butyl]spermine·5HCl (ASPM-5), and N^5,N^{10}-bis(4-carboranylbutyl)spermine·4HCl (SPM-5,10) (Zhuo et al., 1999; Cai et al., 1997). The anionic agents and cationic boronated polyamines were incorporated into liposomes by passive entrapment and remote loading, respectively. Higher cellular uptake was observed with FR-targeted liposomes than with nontargeted liposomes irrespective of the chemical form of the boron and the method used in liposomal preparation. A detailed description of these findings is presented in the following report.

EXPERIMENTAL PROCEDURES

Reagents. BSH was purchased from Centronic Ltd. (Croydon, U.K). Na_3 ($B_{20}H_{17}NH_3$) was kindly provided by Drs. M. Frederick Hawthorne and Kenneth Shelly in the Department of Chemistry at the University of California at Los Angeles; boronated polyamines, including the two spermidine derivatives N^5-(4-carboranylbutyl)spermidine·3HCl (SPD-5) and N^5-[4-(2-aminoethyl-*o*-carboranyl)-butyl]spermidine·4HCl (ASPD-5), and the three spermine derivatives N^5-(4-carboranylbutyl)spermine·4HCl (SPM-5), N^5-[4-(2-aminoethyl-*o*-carboranyl)butyl]spermine·5HCl (ASPM-5), and N^5,N^{10}-bis(4-carboranylbutyl)spermine·4HCl (SPM-5,10) were synthesized in our laboratories as reported previously (Zhuo et al., 1999; Cai et al., 1997). Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Alabaster, AL). PEG900-Cholesterol (PEG-Chol) was purchased from Fluka Chemical Co. (Milwaukee, WI). Folic acid and cholesterol (Chol) were purchased from Sigma Chemical Co. (St. Louis, MO). Folate-free RPMI 1640 medium and other tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY). BCA Protein Assay Kit was purchased from Pierce Chemical Co. (Rockford, IL). Folate–poly(ethylene glycol) (MW ~ 3350)–distearoyl phosphatidylethanolamine (folate–PEG–DSPE) was synthesized as described previously (Lee and Low, 1995). The final folate–PEG–DSPE product was purified on a silica gel (70–200 mesh) using a solvent gradient of 15 to 80% methanol in CH_2Cl_2. Product purity was confirmed by thin-layer chromatography analysis on silica gel GF (75:36:6 chloroform/methanol/water), as described previously (Gabizon et al., 1999) and showed a single spot ($R_f = 0.61$).

Cell Culture. KB human cancer cells (American Type Culture Collection # CCL 17), derived from an epidermal carcinoma of the oral cavity, were obtained as a gift from Dr. Philip S. Low at Purdue University (West Lafayette, IN). The cells were maintained in folate-free RPMI 1640 medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 5% fetal bovine serum, which provides the only source of folate. The cells were cultured as a monolayer in a humidified atmosphere containing 5% CO_2 at 37 °C.

Liposome Preparation. Small unilamellar vesicles (SUVs) were prepared by high-pressure polycarbonate membrane extrusion, as previously described (Lee and Low, 1995). Briefly, a lipid mixture of the desired composition (including folate–PEG–DSPE for the FR-targeted formulation), containing 30 mg of total phospholipids, was dissolved in chloroform, dried into a thin film under a stream of nitrogen, and then desiccated under vacuum for another 30 min. Boronated agents then were loaded into liposomes by either passive entrapment (during liposome formation) or remote loading (after liposome formation), as described in the following sections.

Passive Entrapment. The dried lipids were hydrated in a 0.5 mL of aqueous solution containing the boronated compound (5 mg/mL BSH or 16.5 mg/mL Na_3 ($B_{20}H_{17}$-

NH_3)). To facilitate lipid hydration, the mixture was subjected to vortex mixing followed by brief sonication in a bath sonicator and six cycles of freezing and thawing, which resulted in the formation of a heterogeneous population of lipidic vesicles. These were manually extruded six times through a 100-nm pore-size polycarbonate membrane using a handheld LiposoFast extruder (Avestin Inc., Ottawa, Canada) attached to two 0.5-mL gastight syringes. The resulting SUVs were purified from nonentrapped molecules by size exclusion chromatography on a 10 mL Sepharose CL-4B column (Sigma Chemical Co., St. Louis, MO) preequilibrated in a buffer consisting of 10 mM sodium phosphate buffer and 150 mM NaCl (phosphate-buffered saline, PBS, pH 7.4). The liposomes were eluted in the void volume fractions whereas the free boronated compounds were eluted in later fractions. Boron concentrations in each of the fractions were quantified by means of direct current plasma-atomic emission spectroscopy (DCP-AES) using a Spectraspan VB spectrometer (Applied Research Laboratories, La Brea, CA), as previously described (Barth et al., 1991). Encapsulation efficiencies were determined by calculating the total boron content in the liposomal fractions and the total boron eluted from the column, including both the liposomal and the free boron fractions, as indicated in the following equation:

$$\% \text{ encapsulation} = \text{liposomal boron}/(\text{liposomal boron} + \text{free boron}) \times 100\%$$

The mean diameter and size distribution of the liposomes were determined by photon correlation spectroscopy on a NICOMP 370 submicron particle analyzer. Phospholipid concentration was measured by an ammonium ferrothiocyanate partitioning colorimetic assay, as previously described (Stewart, 1980). The mean particle diameters were between 100 and 150 nm for all liposome preparations. Liposome samples were stored at 4 °C and used within 2 weeks of preparation, during which time no significant leakage (<1%) of the entrapped contents was detected by gel-filtration fractionation on a Sepharose CL-4B column.

Remote Loading. Boronated polyamine derivatives (SPD-5, ASPD-5, SPM-5, ASPM-5, and SPM-5,10) were incorporated into preformed liposomes via a transmembrane pH gradient. Briefly, SUVs were prepared using the above-described method by hydrating them in either 300 mM citric acid (pH 4.0) or 250 mM ammonium sulfate. Boron loading was accomplished by incubating the liposomes with the boronated polyamines, which each contained 1.1 mg (or ~0.1 mmol) of boron dissolved in HEPES buffered saline (HBS, 150 mM NaCl, 20 mM HEPES, pH 7.4), for 60 min at 60 °C. Liposomes then were separated from the free drug by gel-filtration on a Sepharose CL-4B column and characterized as described above.

Uptake of Boronated Liposomes by Cultured KB Cells. Monolayer cultures of KB cells in a logarithmic growth phase were disaggregated by a short exposure to 0.5% trypsin. The cells then were sedimented by centrifugation at 500*g*, resuspended in folate-free RPMI 1640 media, divided into aliquots of 1×10^6 cells, and incubated with folate-derivatized or nontargeted control boronated liposomes for 2 h at 37 °C with gentle shaking. The number of cells was determined by counting using a hemocytometer. The cells then were sedimented at 500*g* and washed 2× with PBS, and the boron concentration in the supernatant and the cell pellet was measured by DCP-AES, as previously described (Barth et al., 1991).

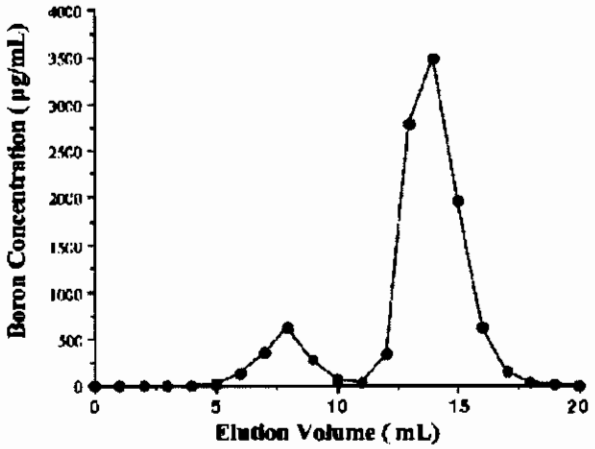

Figure 2. Size-exclusion chromatographic fractionation of L-[Na_3 ($B_{20}H_{17}NH_3$)] on a Sepharose CL-4B column. One milliliter of polycarbonate membrane extruded liposomes was loaded on to an 11-mL column, which had been prewashed with three volumes of PBS (pH7.4). Boron content in each elution fraction was determined by DCP-AES, as described in Experimental Procedures.

For FR blocking studies, the cells were incubated with boronated liposomes in the presence of 1 mM free folic acid. Cellular uptake was normalized to micrograms of boron per 10^9 cells by multiplying by the appropriate factor.

RESULTS

Liposomal Entrapment of BSH and $Na_3(B_{20}H_{17}NH_3)$. Boronated liposomes (SUVs), composed of egg PC/Chol/PEG-Chol (60:35:5, mole/mole), were prepared by lipid hydration in a solution of the anionic hydrophilic boron agent BSH or $Na_3(B_{20}H_{17}NH_3)$, followed by extrusion through a 100-nm pore-size polycarbonate membrane, as described in the Experimental Procedures. The 5 mol % PEG−Chol was incorporated in the liposome formulation to provide steric stabilization against plasma protein binding during systemic administration for future in vivo studies. Egg PC/Chol/PEG−Chol/folate−PEG−DSPE (60:35:4.5:0.5, mole/mole) was used in the preparation of FR-targeted liposomes. The liposome samples were fractionated on a Sepharose CL-4B column. As shown in a typical chromatogram (Figure 2), liposomal $Na_3(B_{20}H_{17}NH_3)$ (L-[$Na_3(B_{20}H_{17}NH_3)$]) could be efficiently separated from the unentrapped free drug using this method. The majority of the boron detected by DCP-AES was eluted in the low molecular weight fractions. Entrapment efficiencies, obtained for BSH and $Na_3(B_{20}H_{17}NH_3)$, were 6% and 15%, respectively. No significant differences were observed between the loading efficiency of FR-targeted and the nontargeted control liposomes (data not shown).

Liposomal Entrapment of Boronated Polyamines. Liposome loading was carried out by remote-loading methods based on the generation of a transmembrane pH gradient, as described in the Experimental Procedures. Either 300 mM sodium citrate (pH 4) or 250 mM ammonium sulfate was used as an intraliposomal trapping agent, which forms an insoluble complex with the boronated polyamines that have diffused across the lipid bilayer into the aqueous core of the liposomes. Identical lipid compositions, with or without the incorporation of folate−PEG−DSPE, were used, as described above for liposomes entrapping anionic boronated agents.

Table 1. The Entrapment Efficiency of Boronated Polyamines by Remote Loading (%)

boronated polyamine derivatives	trapping agent	
	sodium citrate	$(NH_4)_2SO_4$
SPD-5	30	93
ASPD-5	26	72
SPM-5	19	26
ASPM-5	13	45
SPM-5, 10	75	85

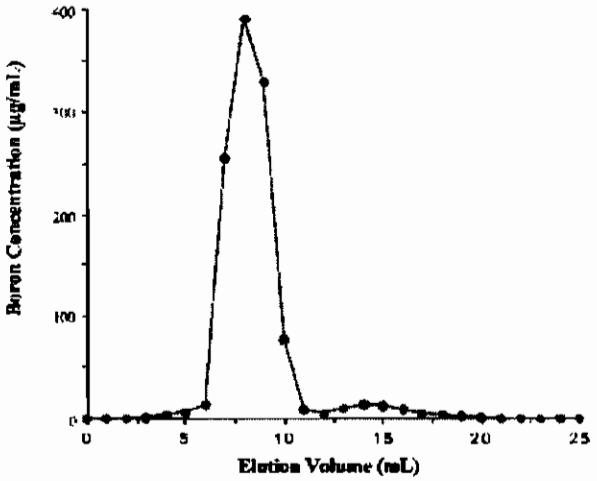

Figure 3. Size-exclusion chromatographic purification of L-[ASPD-5] on a Sepharose CL-4B column. One milliliter of polycarbonate membrane extruded liposomes was loaded on to an 11-mL column prewashed with three volumes of PBS (pH7.4). Boron content in each elution fraction was determined by DCP-AES, as described in Experimental Procedures.

The loading efficiencies were highly variable and ranged from 13% to 93%, as shown in Table 1. The boronated liposomes were easily separated from the free compound, as shown in a representative size-exclusion chromatogram of liposomes entrapping ASPD-5 (Figure 3). Comparing the various boronated polyamines with a single carborane moiety (SPD-5, ASPD-5, SPM-5, ASPM-5), the spermidine derivatives showed a higher entrapment efficiency compared to the spermine derivatives. Among the spermine derivatives, SPM-5,10, which has two attached carborane cages, showed a higher entrapment efficiency than SPM-5, which contains a single carborane moiety. Finally, liposome loading using ammonium sulfate as the trapping agent resulted in an overall higher efficiency of boron entrapment compared to procedures using sodium citrate as the trapping agent. Therefore, all liposomes containing the boronated polyamines used in subsequent tumor cell uptake studies were prepared using ammonium sulfate as the trapping agent. Incorporation of folate−PEG−DSPE did not significantly influence liposomal loading efficiency (data not shown).

Cellular Uptake of FR-Targeted Boronated Liposomes. To study the potential use of folate-derivatized liposomes as tumor cell selective delivery agents for BNCT, cellular binding of the liposomes was evaluated using cultured KB cells, which express amplified FR. A representative concentration dependent cellular uptake curve using liposome-entrapped BSH is shown in Figure 4. Greater cellular uptake of boron was obtained with folate-derivatized liposomal BSH (f-L-[BSH]) compared to the nontargeted control liposomes (L-[BSH]). Furthermore, the FR-targeted liposomes showed binding saturation at boron concentrations greater than 4 μM and this

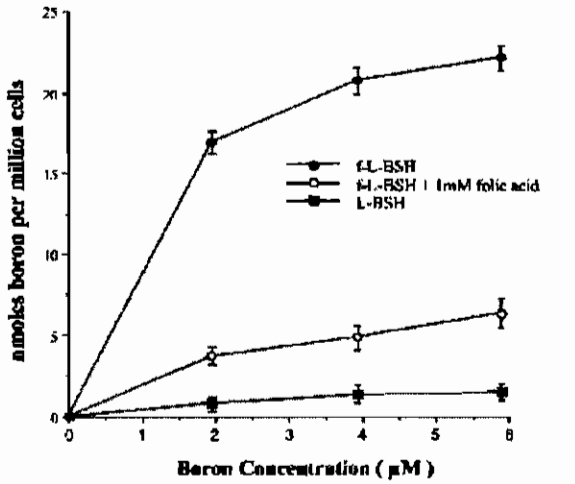

Figure 4. FR-mediated uptake of liposomal BSH in cultured KB cells. Tumor cells were suspended with trypsin and incubated with BSH-containing liposomes at the indicated boron concentrations for 2 h at 37 °C. Tumor cell associated boron was analyzed as described in Experimental Procedures.

was inhibited by 1 mM folic acid, suggesting that the uptake of f-L-BSH was dependent upon FR expression. The maximum boron uptake obtained with f-L-[BSH] was ~240 μg/10^9 cells.

Cellular uptake of boron, following a 2 h incubation at 37 °C with a variety of boronated agents, each containing 4 μM total boron, entrapped in either FR-targeted (f-L-[boron]) or nontargeted control liposomes (L-[boron]), are summarized in Table 2. There was a 2.3- to 49-fold higher boron uptake in cells treated with the f-L-[boron] compared to cells treated with L-[boron]. Furthermore, uptake of f-L-[boron] could be blocked by coincubation with 1 mM folic acid, indicating that cellular uptake was FR dependent. Boron delivery to KB cells generally was more efficient with f-L-[boron] containing the cationic polyamine derivatives compared to the liposomes containing the anionic boronated species. The maximum cellular uptake of f-L-[boron] was variable and was as high as 1584 μg per 10^9 cells with liposomes entrapping SPM-5,10.

DISCUSSION

In the present study, we have evaluated the incorporation of a series of carborane cage-containing derivatives into FR-targeted liposomes. Using either passive or remote loading techniques, the efficiency of loading and the cellular uptake of boron have been determined. Anionic carborane derivatives BSH and $Na_3(B_{20}H_{17}NH_3)$, which have very high boron content and are membrane impermeable due to their high degree of ionization (Hawthorne and Shelly, 1997), were loaded into SUVs by passive entrapment. In the absence of significant bilayer interactions, the liposomal entrapment efficiencies for water-soluble agents primarily will be determined by the percent entrapped volume, provided that solute concentrations reach equilibrium across the lipid bilayer during liposome preparation. Under these conditions, higher percentage entrapment can be achieved by (i) increasing the lipid concentration, (ii) adopting procedures to ensure equilibration of solute across the membrane, and (iii) increasing the mean vesicle diameter. A relatively small particle size is required for systemic liposomal drug delivery in order to enhance diffusion into the tumor. Therefore, we have selected a polycarbonate

Table 2. Uptake of Liposomal Boron Compounds by Cultured KB Cells

boron compounds	boron-to-lipid ratio × 10^3 (wt/wt)	f-L-[boron][a]	L-[boron][a]	f-L-[boron] + 1 mM folic acid
BSH	3.0	231 ± 40 [b]	14 ± 2	53 ± 10
$Na_3(B_{20}H_{17}NH_3)$	28	108 ± 26	2.2 ± 1.1	18 ± 12
SPD-5	34	671 ± 154	76 ± 8	297 ± 26
ASPD-5	26	770 ± 143	75 ± 30	352 ± 44
SPM-5	9.5	132 ± 21	17.6 ± 3	33 ± 3
ASPM-5	17	154 ± 29	67 ± 9	132 ± 18
SPM-5,10	31	1584 ± 56	154 ± 19	1331 ± 74

[a] f-L-[boron] designates the FR-targeted boron containing liposomes; L-[boron] designates the nontargeted control liposomes. [b] Boron concentrations were determined by means of DCP-AES. The values indicated were micrograms of boron per 10^9 cells ± standard deviation, based on three replicate samples for each compound.

membrane extrusion-based procedure to generate SUVs with mean diameters in the range of 100−150 nm. The entrapment efficiencies obtained for BSH and $Na_3(B_{20}H_{17}NH_3)$ (6% and 15%) were substantially higher than those reported previously (Mehta et al., 1996; Feakes et al., 1994). The lower entrapment efficiencies observed by Feakes et al. possibly could have been due to the small size of the liposomes (~60 nm), although differences in lipid compositions and the procedure used to prepare these liposome preparation also may have played a role. The difference in encapsulation efficiency of BSH and $Na_3(B_{20}H_{17}NH_3)$ may be due to differences in their binding affinity for the lipid bilayer. The incorporation of folate−PEG−DSPE, a targeting ligand for the FR, did not significantly affect liposomal loading efficiency. Further enhancement in entrapment efficiency might be achieved by increasing the lipid concentration during lipid hydration. Although much greater incorporation efficiencies can be achieved using lipophilic derivatives of carboranes, their high lipophilicity would reduce subcellular localization to the nucleus. A combination of encapsulation of hydrophilic boron agents with the incorporation of lipophilic carborane derivatives into the bilayer would maximize liposomal boron content and simultaneously allow the targeting of multiple intracellular targets. Further evaluation of this approach is warranted.

Boronated polyamines have been designed to target DNA following cellular uptake (Cai et al., 1997). The amphiphilicity and weakly basic properties of these agents make them suitable candidates for remote loading into liposomes. In contrast to passive entrapment, remote loading is achieved based on the bilayer permeability of boronated polyamines at basic pH and the formation of insoluble complexes between these agents and a trapping agent, which consist of either sodium citrate or ammonium sulfate. Remote loading procedures have been applied to anthracyclines, resulting in close to 100% entrapment (Muggia, 2001). In the present study, we compared the effectiveness of 300 mM sodium citrate and ammonium sulfate as trapping agents during remote loading. The latter was found to be more effective, possibly due to its greater capacity to form stable complexes with the boronated polyamines. Remote loading of weakly basic compounds into liposomes is presumably mediated by precipitation of the agent inside the liposome, either with citrate or sulfate as the counterion. Neither citrate nor sulfate ions are expected to cross the lipid bilayer, either alone or as a polyamine complex. Therefore, the observed differences in loading efficiency were possibly due to differences in intraliposomal pH values and the solubility product (K_{sp}) values for the polyamines with each type of counterions.

The liposomal loading efficiency also appeared to be influenced by the chemical structure of the boron derivatives. When only one carborane cage was present, higher percentages of boron encapsulation were observed when the ionic component was spermidine (SPD-5, ASPD-5) compared to those obtained with spermine (SPM-5, ASPM-5). It is possible that at a weakly basic pH the additional ionizable amine component and the corresponding increase in overall size of the molecule impeded diffusion of spermine derivatives across the phospholipid bilayer. In addition, the presence of two carborane cages (in SPM-5,10), which increased the lipophilicity of the molecule, appeared to facilitate liposome loading, suggesting that lipophilicity might be an additional determining factor for optimizing remote loading.

Based on the premise that cholesterol formed a stable lipophilic anchor for PEG, PEG900-Chol was incorporated into the liposomes to enhance their colloidal stability. A previous report (Beugin et al., 1998) has shown that PEG-Chol derivatives have lower critical micelle concentration (CMC) values than the corresponding PEG-PE derivatives and were stably incorporated into liposomes with similar efficiency and stability. In addition, Ishiwata et al. (1995) reported that PEG−Chol derivatives could prolong systemic circulation time of liposomes. Furthermore, our previous studies have shown that folate−PEG−Chol effectively targeted liposomes to tumor cells expressing amplified level of folate receptor (Guo and Lee, 2000).

Receptor-mediated targeting of boronated liposomes potentially may improve the delivery of boron to tumor cells. FR-targeted liposomes can be easily prepared through the incorporation of folate−PEG−DSPE, a lipophilic derivative of FR ligand folic acid. A proposed mechanism of FR-mediated intracellular boron delivery is shown in Figure 5. Compared to polypeptide-based targeting ligands, folate is small in size, readily available, easily amenable to liposomal incorporation, highly specific for FR positive tumor cells, nonimmunogenic, and selective for a cellular marker that has very limited normal tissue distribution and is widely overexpressed in a wide variety of tumors, including over 90% ovarian carcinomas (Garin-Chesa et al., 1993; Ross et al., 1994; Weitman et al., 1992). Boron uptake by FR-positive KB cells was much greater with FR-targeted f-L-[boron] liposomes compared to the nontargeted control liposomes. The variability in the boron delivery efficiencies of f-L-[boron] partly may be attributable to differences in boron loading efficiencies and boron-to-lipid ratios of the various liposomal preparations. Uptake of f-L-[boron] by KB cell was only partially blocked by 1 mM folic acid, which may have been due to increased binding affinity resulting from multivalent interactions between liposomes and cellular FRs.

The ultimate purpose of developing FR-targeted boronated liposomes is to selectively deliver sufficient quantities of boron to FR-expressing tumor cells. Comparison

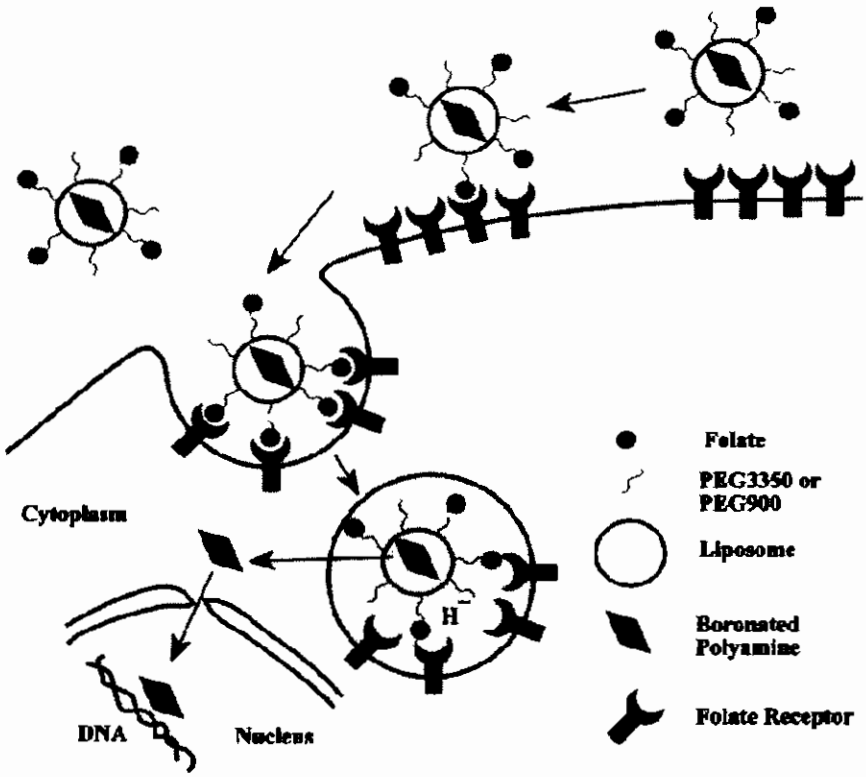

Figure 5. Proposed mechanism of intracellular boron delivery that involves both FR-mediated tumor cell targeting and intracellular DNA targeting. FR-targeted boronated polyamine containing liposomes are shown to be internalized by a cell via FR-mediated endocytosis. The encapsulated boron agent is then released into the cytosol via endosomal escape and bound to the DNA in the cellular nucleus.

of delivery efficiencies by different liposomal formulations, therefore, was based on exposing cells to the same concentrations of boron, rather than lipids. The FR targeting properties of folate-derivatized liposomes have been reported previously (Lee and Low, 1995) and, therefore, was not the main focus of the present study. The alternative would have been to keep the lipid concentration constant, which would result in variable amounts of boron. Results based on this strategy might be difficult to interpret since our data focused on measurements of cell-associated boron. The choice of 4 μM boron concentration in cellular binding studies was based on the finding that higher concentrations of boron and lipids resulted in significant nonspecific cellular uptake (data not shown).

The variability in cellular boron uptake and in FR-targeted-to-nontargeted enhancement ratios were influenced by a number of variables. These included (i) the lipid concentration used in the binding assay, (ii) the boron-to-lipid ratio of the liposomal formulation, and (iii) the ζ-potential of the liposomal surface, which was affected by binding of the cationic and lipophilic boronated polyamines. Data on cellular boron uptake indicated that boronated polyamine liposomal formulations with higher boron-to-lipid ratios generally had greater nonspecific cellular uptake. This suggests that uptake of FR-targeted liposomes may have been due to both receptor-mediated and nonspecific binding, which would increase with elevated ζ-potential due to greater liposome surface binding of the boronated polyamines.

In vivo tumor localization of boronated liposomes potentially can be affected by passive targeting due to increased endothelial permeability and limited lymphatic drainage frequently observed in solid tumors (Matsumura et al., 1986). Tumor-targeted immunoliposomes and FR-targeted liposomes have shown moderate enhancement in tumor accumulation compared to nontargeted liposomes, but the degree of enhancement was highly variable (Ishida et al., 2001; Park et al., 1997; Guo et al., 2000). For example, transferrin receptor-targeted immunoliposomes showed only a 2-fold increase in boron localization in a rat xenograft model (Maruyama et al., 1999; Ishida et al., 2001). Intratumoral and subcellular distribution of the boron are important determinants for the effectiveness of neutron capture therapy (Hartman et al., 1994). It is conceivable that FR-targeted liposomes may be more efficiently internalized by FR-bearing tumor cells following extravasation compared to nontargeted liposomes within the solid tumor.

In summary, we have shown that FR-targeted liposomes can deliver larger amounts of boron (up to 1584 μg/10^9 cells) to receptor positive tumor cells in vitro than any other delivery agent currently being investigated in our laboratories. This amount greatly exceeds the boron concentration (20−35 μg per gram of tumor) required to sustain a lethal $^{10}B(n,\alpha)^7Li$ capture reaction for BNCT. Boron delivery was selective to FR expressing cells since it can be blocked by excess free folate. Folate-derivatized liposomes containing boronated polyamines would be particularly attractive targeting agents since they also could deliver boron to cellular DNA. Further studies are warranted to evaluate the in vivo tumor localizing potential of these boron carriers in FR-positive tumor cells and their utility for BNCT.

ACKNOWLEDGMENT

This work has been supported by the National Institute of Health grant R01CA79758 and Department of Energy grant DE-FG02-98ERG295.

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BC015557Y

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Efficient intracellular drug and gene delivery using folate receptor-targeted pH-sensitive liposomes composed of cationic/anionic lipid combinations

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Received 28 November 2001; accepted 9 January 2002

Abstract

pH-sensitive liposomes are designed to promote efficient release of entrapped agents in response to low pH. In this study, novel pH-sensitive liposomes consisting of cationic/anionic lipid combinations are evaluated for intracellular drug and gene delivery. First, liposomes composed of egg phosphatidylcholine, dimethyldioctadecylammonium bromide (DDAB), cholesteryl hemisuccinate (CHEMS), and Tween-80 (25:25:49:1, mol/mol) were shown to stably entrap calcein at pH 7.4 and undergo rapid content release and irreversible aggregation under acidic pH. Compared to pH-sensitive liposomes incorporating dioleoylphosphatidylethanolamine, these liposomes showed improved retention of pH-sensitivity in the presence of serum. The folate receptor (FR), which is amplified in a wide variety of human tumors, could be targeted by incorporating 0.1 mol% folate-polyethyleneglycol-phosphatidylethanolamine (f-PEG-PE) into liposomes. f-PEG-PE has been shown to facilitate FR-mediated endocytosis of liposomes into KB human oral cancer cells, which express amplified FR. FR-targeted pH-sensitive liposomes produced increased cytosolic release of entrapped calcein, as shown by fluorescence microscopy, and enhanced cytotoxicity of entrapped cytosine-β-D-arabinofuranoside, as shown by an 11-fold reduction in the IC_{50} in KB cells, compared to FR-targeted non-pH-sensitive liposomes. Furthermore, FR-targeted pH-sensitive liposomes composed of DDAB/CHEMS/f-PEG-PE, combined with polylysine-condensed plasmid DNA, were shown to mediate FR-specific delivery of a luciferase reporter gene into KB cells in the presence of 10% serum. These findings suggest that cationic lipid-containing pH-sensitive liposomes, combined with FR targeting, are effective vehicles for intracellular drug and gene delivery.

Keywords: pH-sensitive liposomes; Folate receptor; Drug targeting; Cytosine-β-D-arabinofuranoside; Gene therapy

1. Introduction

Liposomes are phospholipid bilayer vesicles with potential application in drug and gene delivery. Cellular uptake of liposomes generally follows an endocytic pathway. pH-sensitive liposomes are designed to undergo rapid destabilization in acidic environments like those found in endocytic vesicles [1]. These liposomes are typically composed of a neutral cone-shaped lipid dioleoylphosphatidyl-

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0168-3659/02/$ – see front matter
PII: S0168-3659(02)00017-2

ethanolamine (DOPE) and a weakly acidic amphiphile, such as cholesteryl hemisuccinate (CHEMS) [2,3]. A key limitation for DOPE-based pH-sensitive liposomes is the loss of acid-induced fusogenicity in the presence of serum, possibly due to interactions between the DOPE-rich lipid bilayer and serum opsonins. Therefore, development of novel liposomal formulations that retain pH-sensitivity in the presence of serum is necessary if pH-sensitive liposomes are to be adopted in an in vivo setting.

In a step toward this goal, Hafez et al. [4] recently reported the preparation of non-DOPE-based pH-sensitive liposomes composed of combinations of cationic and anionic lipids including dioleoyldimethylammonium chloride (DODAC) combined with cholesteryl hemisuccinate (CHEMS) and 3-α-[*N*-(*N'N'*-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) combined with dioleoylphosphatidic acid (DOPA). These liposomes carried net negative charges at neutral pH and contain a titratable lipid component consisting of either a weakly acidic (anionic) or a weakly basic (cationic) amphiphile. The lipids formed a stable bilayer at neutral or basic pH. At acidic pH, where the weakly acidic or the weakly basic amphiphile within the bilayer was partially protonated, the liposomes became charge neutral and underwent rapid aggregation and membrane fusion due to the removal of electrostatic colloidal stabilization. The pH required for triggering liposomal fusion was dependent on the cationic to anionic lipid ratio and the pK_a of the titratable component (e.g. CHEMS and DC-Chol) in the liposomes [4]. Liposomes composed solely of charged lipids, however, may not be suitable for drug delivery since they do not form vesicles that are capable of stably entrapping drug molecules.

Since pH-sensitive liposomes can facilitate the cytosolic release of membrane impermeable molecules, it might be feasible to combine their use with a targeting ligand that promotes receptor-mediated endocytosis. The folate receptor (FR) is a 38-kDa glycosyl phosphatidylinositol (GPI)-anchored glycoprotein with highly restricted normal tissue distribution and amplified expression in a wide variety of human tumors including over 95% of non-mucinous ovarian carcinomas [5–7]. Liposomes incorporating a lipophilic folate derivative, such as folate-PEG-DSPE or folate-PEG-Chol, have been shown to efficiently deliver antitumor agents into FR-bearing tumor cells via receptor-mediated endocytosis [8–11]. Therefore, combining pH-sensitive formulations with FR targeting could potentially improve drug or gene delivery to tumor cells possessing amplified levels of FR.

In this report, two novel liposomal formulations were investigated with pH-sensitive formulations consisting of cationic/anionic lipid combinations and including an FR targeting ligand for pH-dependent destabilization and for drug and gene delivery efficiency in tumor cells with amplified FR expression [11]. Our findings indicate that liposomes based on cationic/anionic combinations and FR targeting are highly efficient vehicles for intracellular drug and gene delivery.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (PC) and dioleoylphosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Poly-L-lysine (PLL, M_r ~29,000), calcein, cholesteryl hemisuccinate (CHEMS), cytosine-β-D-arabinofuranoside (araC), dimethyldioctadecylammonium bromide (DDAB), distearoylphosphatidylethanolamine (DSPE), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), folic acid dihydrate, polyoxyethylenesorbitan monooleate (Tween-80), Sepharose CL-4B resin and Triton X-100 were purchased from Sigma (St. Louis, MO). Folate-polyethyleneglycol (MW ~3,350)-distearoylphosphatidylethanolamine (f-PEG-PE) was synthesized as described previously [9]. Luciferase assay reagents were obtained from Promega (Madison, WI). Polycarbonate membranes and a handheld LiposoFast™ lipid extruder were obtained from Avestin (Ottawa, Canada). BCA protein assay reagents were purchased from Pierce Chemical (Rockford, IL). Tissue culture media were purchased from Life Technologies (Rockville, MD).

2.2. Cell culture

KB human oral carcinoma cells, which exhibit amplified FR expression, were kindly provided by Dr

Philip Low at Purdue University (West Lafayette, IN). The cells were cultured as monolayers in 75-cm^2 flasks in folate-free RPMI 1640 media supplemented with antibiotics and 10% FBS in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Liposome preparation

Liposomes were prepared using a previously described polycarbonate membrane extrusion method [12]. Briefly, a chloroform solution of a lipid mixture with the desired lipid composition was dried into a thin film on the wall of a glass tube under a stream of nitrogen gas. Residual chloroform was removed by vacuum desiccation for an additional 60 min. The lipids were then resuspended in a buffer solution containing the molecule of interest, such as calcein or araC, by vortexing and periodic sonication in a bath sonicator (model 50 HT, VWR Scientific Product). The lipidic suspension was then subjected to six cycles of freezing and thawing followed by ten cycles of extrusion through a 100-nm pore-size polycarbonate membrane using a handheld LiposoFast™ extruder. FR-targeted liposomes were prepared by including 0.1 mol% of f-PEG-PE in the initial lipid mixture [9]. Un-entrapped drug was separated from the liposomes by gel filtration on a 10-ml Sepharose CL-4B column equilibrated in phosphate buffered saline (PBS, 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). Drug concentration in the liposome preparations was determined by measuring absorption (at 495 nm for calcein and 273 nm for araC) of the liposomes dissolved in methanol/water (3:1, v/v) based on the molar extinction coefficient of the free drug. Liposome mean diameter and size distribution were determined by photon correlation spectroscopy using a NICOMP Particle Sizer Model 370.

2.4. Calcein dequenching assays

Liposomal formulations were evaluated for their ability to stably entrap membrane impermeable molecules by determining the percentage of liposomal calcein release of various formulations following a 2-week incubation at 4 °C or 10 min at 37 °C in the presence of 10% fetal bovine serum (FBS) determined by fluorescence dequenching, as previously described [13]. Furthermore, the liposomes were characterized for low pH-triggered calcein release using the following procedure. Freshly column-purified liposomes entrapping 80 mM calcein and containing 50 nmol of lipid were added to 2 ml PBS or sodium acetate buffer (100 mM NaCl, 10 mM Na acetate, pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5) with or without 90% FBS in disposable cuvettes. After a 10-min incubation at 37 °C, buffer pH was adjusted to pH 7.4 and calcein fluorescence was measured before and after addition of 0.15% Triton X-100, which defines 100% calcein leakage from the liposomes. The percentage of low pH-triggered calcein release was calculated using the formula below:

$$\%\ \text{low pH-triggered calcein release} = ((F_{pH} - F_{7.4})/(F_{TX} - F_{7.4}))^{*}100\%$$

where F_{pH} is fluorescence intensity following incubation in the low pH buffer, $F_{7.4}$ is fluorescence intensity after incubation at pH 7.4 and F_{TX} is fluorescence intensity after the addition of Triton X-100.

All fluorescence measurements were performed on a Perkin Elmer LS-5B spectrofluorometer operated with FTWinlab software (Morena Valley, CA). The excitation and emission wavelengths were set at 490 and 520 nm, respectively.

2.5. pH-induced liposomal aggregation

Liposomal aggregation in response to reduced buffer pH was measured by increase in particle size. Some 40-μl samples of the liposomes containing 200 nmol of lipid were diluted in 4 ml of sodium acetate buffer of various pH values and incubated at 37 °C. At various time points, aliquots of the samples were withdrawn and the mean particle diameter was determined by photon correlation spectroscopy on a NICOMP Particle Sizer Model 370.

2.6. Uptake of calcein containing liposomes by cultured KB cells

Cellular uptake of calcein-containing liposomes was examined by fluorescence microscopy. KB cells cultured as a monolayer were harvested by a brief treatment with trypsin/EDTA. The suspended KB cells were gently pelleted by centrifugation, resus-

pended in fresh culture media at a density of 10^6 cells/ml, and aliquoted into 1.5-ml centrifuge tubes. A 1-ml aliquot of the cell suspension was incubated for 3 h at 37 °C under gentle shaking with liposomes at a final calcein concentration of 20 μM. For a competitive binding assay, 1 mM free folic acid was included in the incubation media. After the incubation, the cells were washed three times with cold PBS and examined on a Leica DMLS Epifluorescence Microscope with a fluorescein dichroic filter set and a photo attachment. The level of cellular liposomal uptake was quantified based on calcein fluorescence by flow cytometry on a Coulter Elite flow cytometer.

2.7. Cytotoxicity assay

Liposomal drug delivery was assessed by comparing the cytotoxicity of various formulations of liposomal araC and free araC in KB cells. The effect of pH-sensitivity on liposomal araC delivery efficiency was evaluated by comparing pH-sensitive and non-pH-sensitive liposomes that are targeted to the FR. Empty pH-sensitive liposomes in combination with free araC were also included as a control to investigate the possibility of inherent liposomal cytotoxicity. The cytosolic delivery of araC was evaluated in KB cells using an MTT cytotoxicity assay, as described previously [9]. For liposome preparation, 400 mM araC was used to hydrate the dried lipid mixture. Liposomes were then prepared by polycarbonate membrane extrusion as described in Section 2.3.

KB cells were seeded in 96-well plates to reach ~25% confluence at the time of the study. The cells were incubated in triplicate with serial dilutions of the various formulations of liposomal araC or free araC for 2 h at 37 °C in media containing 10% FBS. In free folate blockade studies, 1 mM folic acid was added to the incubation media. The cells were washed four times with PBS (pH 7.4), and fresh media was then added. After an additional 48 h of incubation, MTT was added and cells were incubated for 1 h at 37 °C. The cells were then dissolved in HCl acidified isopropanol, and cellular viability was determined by measuring absorption at 570 nm using an automated plate reader. The concentrations of araC leading to 50% cell kill (IC_{50}) were then derived from the concentration dependent cell viability curves.

2.8. Plasmid DNA preparation

Plasmid containing a firefly luciferase reporter gene under the cytomegalovirus promoter, pCMV-Luc, was obtained as a gift from Dr Leaf Huang at the University of Pittsburgh. The DNA was purified using the Qiagen mega kit (Qiagen, Santa Clarita, CA). Plasmid DNA was quantified by measuring UV absorbance at 260 nm and analyzed for structural integrity and percentage of supercoiled DNA by electrophoresis on a 0.9% agarose gel.

2.9. Preparation of LPDII

pH-sensitive liposomes were prepared as described in Section 2.3 using 20 mM HEPES (pH 8.0) as the buffer of hydration and lipid compositions of DDAB/CHEMS/f-PEG-PE (30:70:0.1) and DDAB/CHEMS/PEG-PE (30:70:0.1) for the targeted and the non-targeted formulation, respectively. For LPDII preparation, 3 μg plasmid DNA was diluted in 60 μl serum-free RPMI 1640 medium and vortexed with an appropriate amount of poly-L-lysine (PLL) dissolved in an equal volume of the same media. Following a 10-min incubation at room temperature, the resulting polyplexes were then mixed with the desired amount of liposomes to prepare LPDII complexes.

2.10. Transfection procedure

Gene delivery efficiency was evaluated using a plasmid construct carrying a luciferase reporter gene. Targeted and non-targeted pH-sensitive liposomes were used as components of an LPDII vector to transfect KB cells with and without the presence of serum. Again, blocking assays were performed with free folate to ascertain that differences seen in targeted liposomes were due to FR targeting. KB cells were seeded in 24-well plates 24 h prior to transfection, which allowed cells to reach 60–80% confluence at the time of transfection. Unless otherwise specified, all transfection experiments were performed in regular growth media containing 10% FBS. Cells were incubated in triplicate with vector

formulations containing 1 μg plasmid DNA in 500 μl culture media for 4 h at 37 °C. The transfection media was replaced with fresh culture media containing 10% FBS and the cells cultured for 24 h. Cells were then rinsed three times with PBS and lysed with a lysis buffer (0.5% Triton X-100, 100 mM Tris–HCl, 2 mM EDTA, pH 7.8). The lysates were centrifuged and the supernatants analyzed for protein content using a BCA protein assay and luciferase activity on a Mini-Lum luminometer (Bioscan, Washington, DC). Relative light unit (RLU) values were converted to picograms of luciferase using a standard curve generated under identical assaying conditions with a firefly luciferase standard. Luciferase activity was presented as relative light units (RLUs) calibrated by cellular lysate protein content, which was determined by the BCA protein assay.

3. Results

3.1. Effect of formulation on the stability of pH-sensitive liposomes

Several lipid compositions, incorporating combinations of cationic lipid DDAB and anionic lipid CHEMS, were evaluated for entrapment of calcein, an anionic membrane impermeable fluorescent dye. Liposomes composed of DDAB/CHEMS (33:66, all ratios are molar ratios) or PC/DDAB/CHEMS (40:20:40) were unable to retain entrapped calcein in the presence of 10% FBS, showing 38% release after a 10-min incubation at 37 °C. Tween-80, which contains a polyoxyethylene headgroup, is known to increase liposomal stability by providing a steric barrier on the surface of liposomes. We evaluated the effect of Tween-80 addition on the stability of the above pH-sensitive liposomes. Liposomes composed of PC/DDAB/CHEMS/Tween-80 (25:25:49:1) showed improved stability at neutral buffer pH, showing only 1.3% calcein release after 2-week storage at 4 °C and 4.1% calcein release after 10-min incubation at 37 °C in buffer containing 10% FBS. Therefore, this liposome formulation was further evaluated for pH-sensitivity and intracellular drug delivery.

3.2. Low pH-triggered calcein release and liposomal aggregation

Liposomes composed of PC/DDAB/CHEMS/Tween-80 (25:25:49:1) and reference control liposomes composed of DOPE/CHEMS (60:40) entrapping 80 mM calcein were evaluated for low pH-triggered calcein release. As shown in Fig. 1, both liposome formulations showed increased calcein release at low buffer pH relative to pH 7.4. However, the presence of 90% serum rendered the DOPE/CHEMS liposomes much less responsive to the lowering of the pH showing only 20–30% additional calcein release. In contrast, PC/DDAB/CHEMS/Tween-80 showed ~55% additional calcein release under the same conditions.

The above liposomes were further evaluated for pH-dependent aggregation. As shown in Fig. 2a, the mean diameter of the liposomes was greatly increased at lower buffer pH values, indicating vesicle aggregation and/or membrane fusion. The time course of liposome aggregation at pH 4.0 is shown in Fig. 2b. The observed particle size increase could not be reversed by adjusting the pH back to 7.4.

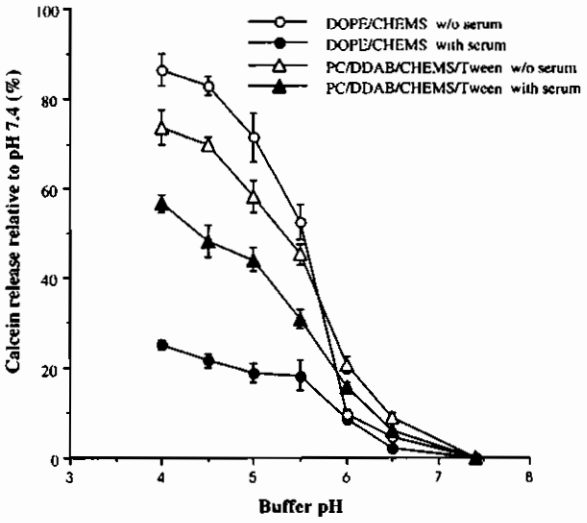

Fig. 1. Calcein release from liposomes triggered by low buffer pH. Calcein-loaded liposomes were incubated in buffers of varying pH, with or without 90% serum, for 10 min at 37 °C. The percentage of calcein dequenching was measured as described in Materials and methods.

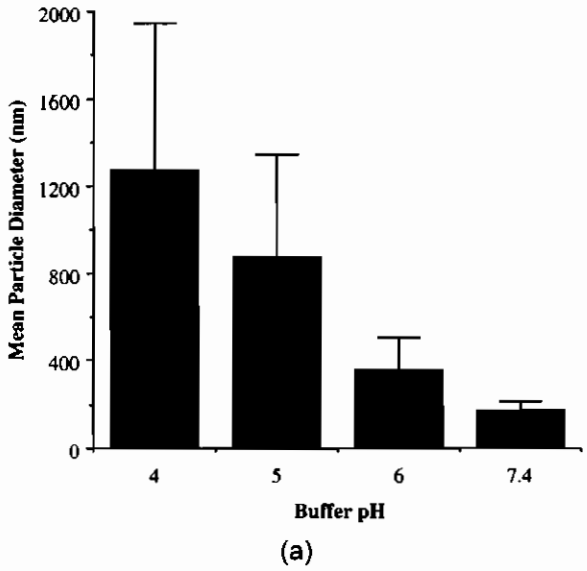

Fig. 2. Increase in liposome size in response to buffer pH. (a) The mean diameter of the liposomes following a 30-min incubation at 37 °C at various buffer pH; (b) time-dependent increase in liposome size at pH 4. The liposome composition used was PC/DDAB/CHEMS/Tween-80 (25:25:49:1); error bar represents 1 S.D.; $n=3$.

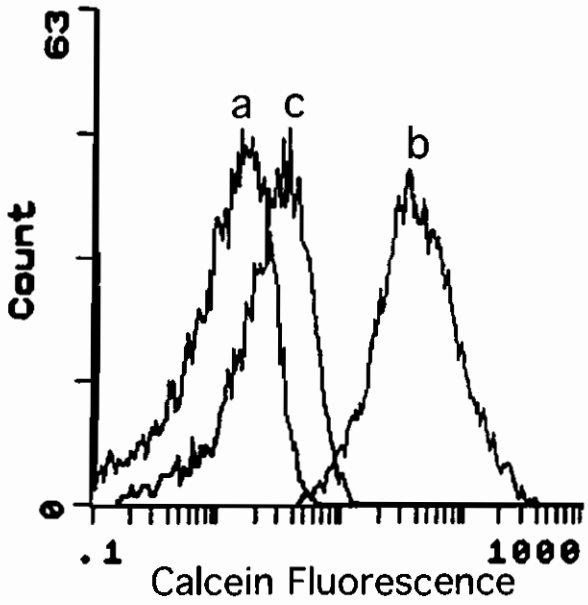

Fig. 3. FR-dependent uptake of PC/DDAB/CHEMS/Tween-80 (25:25:49:1) liposomes entrapping calcein. KB cells were incubated with 20 μM calcein entrapped in (a) non-targeted, (b) FR-targeted liposomes (containing 0.1 mol% f-PEG-PE), or (c) FR-targeted liposomes plus 1 mM free folate for 1 h at 37 °C. Cellular uptake of the liposomes was measured by flow cytometry as described in Materials and methods.

3.3. FR-targeting of pH-sensitive liposomes

Flow cytometry demonstrated, as shown in Fig. 3, that FR-targeted liposomes containing calcein had a 34-fold greater uptake than the non-targeted control liposomes. This increase in liposome uptake could be blocked by 1 mM free folate. Fluorescence microscopy demonstrated differing intracellular fluorescence distributions for targeted pH-sensitive and non-pH-sensitive liposomes. As shown in Fig. 4, KB cells treated with FR-targeted non-pH-sensitive liposomes, composed of PC/Chol/f-PEG-PE (60:40:0.1), had a punctate cytoplasmic distribution indicating endosomal sequestration of calcein. In contrast, cells treated with FR-targeted pH-sensitive liposomes, composed of PC/DDAB/CHEMS/Tween-80/f-PEG-PE (25:25:49:1:0.1), displayed diffuse cytoplasmic fluorescence indicating significant cytosolic release of the fluorescent dye.

3.4. Cytotoxicity of FR-targeted liposomal araC

The IC_{50} for free araC, araC entrapped in pH-sensitive liposomes, non-pH-sensitive liposomes, and free araC plus empty pH-sensitive liposomes were

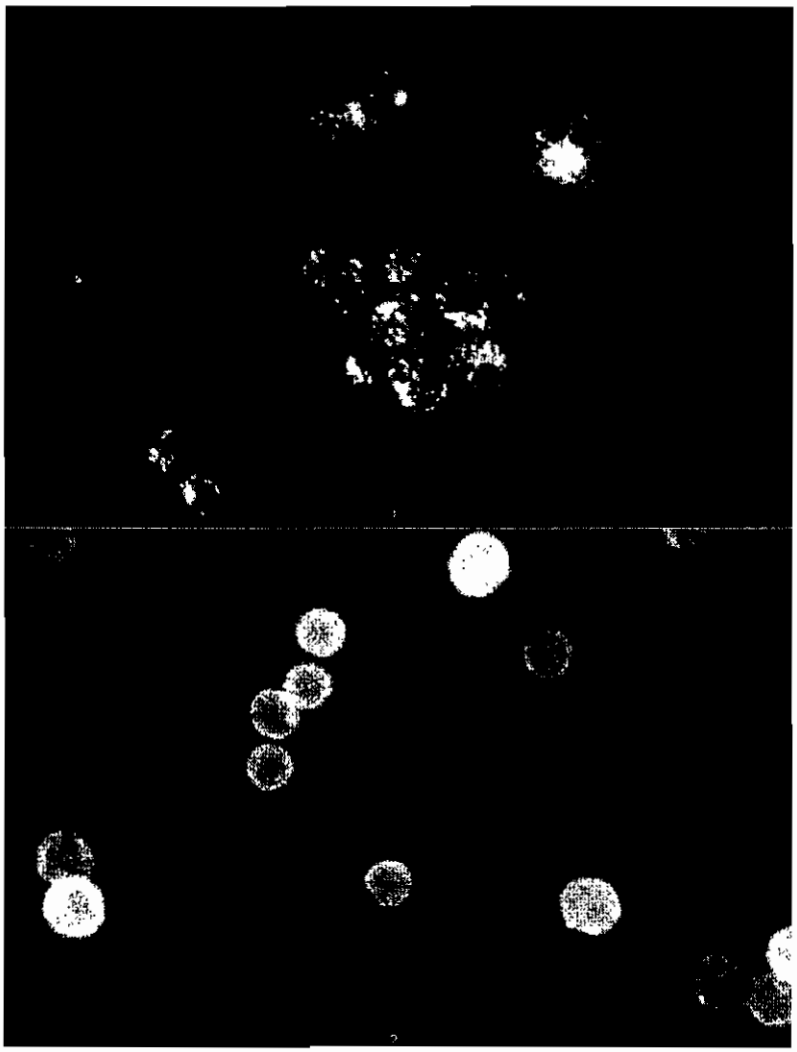

Fig. 4. Fluorescence micrograph of KB cells treated with FR-targeted calcein-containing liposomes. (Upper panel) Cells treated with FR-targeted non-pH-sensitive liposomes composed of PC/CHEMS/f-PEG-PE (60:40:0.1). (Lower panel) Cells treated with FR-targeted pH-sensitive liposomes composed of egg PC/DDAB/CHEMS/Tween-80/f-PEG-PE (25:25:49:1:0.1).

compared for cytotoxicity based on an MTT assay in KB cells. As shown in Table 1, araC showed low cytotoxicity (IC_{50} = 5,625 μM) when added to the cells as a free drug. Entrapment of araC in FR-targeted non-pH-sensitive liposomes increased drug cytotoxicity (IC_{50} = 991 μM). Delivery of araC in FR-targeted pH-sensitive liposomes enhanced cytotoxicity a further 11 times (IC_{50} = 89 μM). In addition, the cytotoxicity of FR-targeted pH-sensitive liposomes was reduced by ~6-fold by 1 mM free folic acid, indicating FR-dependence of the observed cytotoxicity. Furthermore, the cytotoxicity was very low (IC_{50} > 10,000 μM) when araC was delivered in non-targeted pH-sensitive liposomes. Finally, free araC in the presence of empty FR-targeted pH-sensitive liposomes also showed very limited cytotoxicity compared to araC entrapped in FR-targeted pH-sensitive liposomes (IC_{50} of 3,966 vs. 89 μM), indicating that efficient intracellular araC delivery required liposomal encapsulation.

Table 1
Cytotoxicity of araC formulations to KB cells determined by MTT assay

Formulation	IC_{50} (μM) ($n=3$)
AraC in FR-targeted pH-sensitive liposomes	89±17
AraC in FR-targeted pH-sensitive liposomes + 1 mM folic acid	523±150
AraC in FR-targeted non-pH-sensitive liposomes	991±231
AraC in non-targeted pH-sensitive liposomes	>10,000
Free araC	5,620±1130
Free araC + FR-targeted pH-sensitive liposomes	3,970±530

3.5. FR-dependent gene delivery using targeted LPDII vectors

LPDII vectors are composed of a polycation-condensed plasmid DNA core complexed with anionic pH-sensitive liposomes via electrostatic interactions [14]. In the present study, cationic lipid containing pH-sensitive liposomes composed of DDAB/CHEMS/f-PEG-PE (30:70:0.1) were incorporated into the LPDII formulation and investigated as an FR-targeted vector for in vitro gene transfer in KB cells. LPDII vectors prepared using liposomes composed of DDAB/CHEMS/PEG-PE (30:70:0.1) were used as a non-targeted control. The liposomes exhibited acidic pH-triggered aggregation similar to that observed with above-described drug carrying liposomes (data not shown). KB cells were transfected with these vectors carrying a luciferase reporter gene. As shown in Fig. 5, the FR-targeted LPDII vectors displayed a 13-fold greater transfection activity when compared to the non-targeted LPDII vector, which could be blocked by 1 mM free folic acid. In contrast to DOPE-based LPDII vectors, which were inactivated by serum, the cationic lipid-based LPDII vectors showed excellent gene transfer activity in the presence of 10% FBS.

4. Discussion

pH-sensitive liposomes are designed to undergo rapid destabilization in acidic environments such as those inside the endosomal compartment. However, current formulations using DOPE as the fusogenic lipid lose their pH-sensitivity in the presence of serum. Hafez et al. have recently reported pH-sensitive liposome formulations composed of mixtures of cationic and anionic lipids such as DODAC and CHEMS. Lipid mixing assays with these liposomes demonstrated strong pH-dependent membrane fusion, which occurred as a result of liposomal charge neutralization in response to pH reduction [4]. In the present study, liposomes composed of cationic/anionic lipid pairs consisting of DDAB and CHEMS failed to effectively entrap molecules as demonstrated by the 38% calcein release seen after a 10-min incubation at 37 °C. Therefore, we have modified the formulation by adding PC and Tween-80 to improve liposomal stability while maintaining the basic pH-sensitive properties of the cationic/anionic lipid combination. A liposome composition of PC/DDAB/CHEMS/Tween-80 (25:25:49:1) provided optimal liposomal stability as evidenced by the minimal release after 2-week storage at 4 °C.

Both DOPE-based and cationic/anionic lipid-based liposomes exhibited similar pH-sensitive properties in the absence of serum. While serum did reduce calcein release by cationic/anionic lipid liposomes, the effect was not as pronounced as with the DOPE-based liposomes. At pH 4.0, cationic/anionic lipid-based liposome calcein release dropped from 75 to 55% with the presence of 90% FBS, while DOPE-based liposome calcein release was reduced from 85 to only 25%. Therefore, the cationic/anionic lipid-based liposomes exhibited superior 'serum-stability' compared to traditional DOPE-based pH-sensitive liposomes. Furthermore, these findings showed that pH-triggered destabilization of the cationic/anionic liposomes was also not abolished by the incorporation of moderate amounts of PC and Tween-80.

In response to acidic buffer pH, the mean particle size became progressively larger as pH decreased and could not be reduced by adjustment of pH back to 7.4. This permanent change in particle size with the decrease in pH suggests the occurrence of

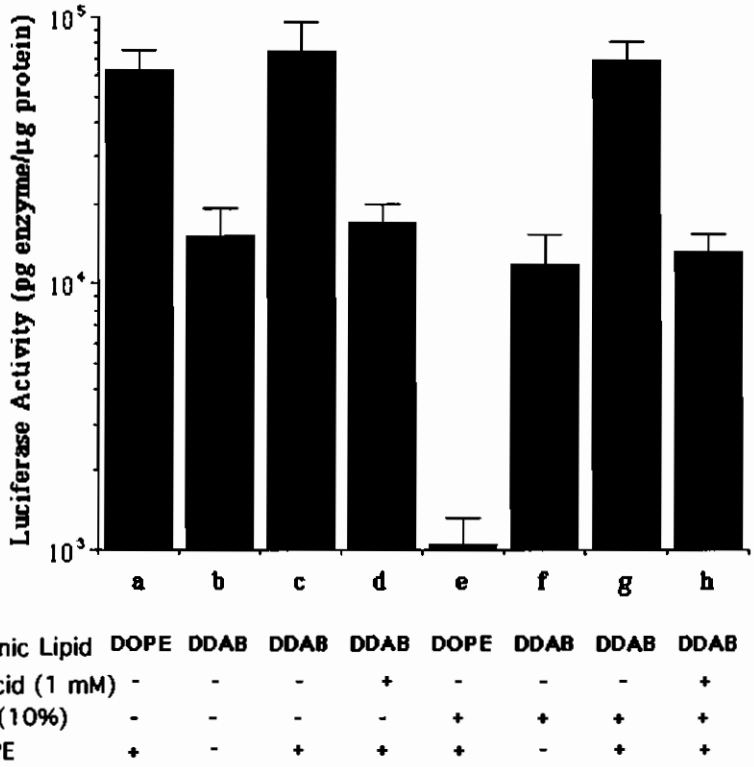

Fig. 5. Gene transfer properties of FR-targeted LPDII vectors in KB cells. KB cells were transfected with LPDII vectors carrying a luciferase reporter gene composed of a PLL/DNA (with a 1.5 to 1 weight ratio) core and pH-sensitive liposomes with the indicated composition at a lipid-to-DNA ratio of 12:1, as described in Materials and methods. a–d were carried out in serum free media; e–h were carried out in media containing 10% FBS. The liposome compositions were: a and e, DOPE/CHEMS/f-PEG-PE (60:40:0.1); b and f, DDAB/CHEMS (30:70); c, d, g, and h, DDAB/CHEMS/f-PEG-PE (30:70:0.1); d and h, transfection was performed in media containing 1 mM free folate ($n=3$).

membrane fusion. At pH 4.0, particle size increased rapidly within the first 10 min of incubation and continued to increase at a slower rate upon prolonged incubation. These trends in pH-dependent particle size were similar to those observed with the DOPE-based liposomes (data not shown).

Besides pH-induced membrane destabilization, the presence of cationic lipids in the liposome bilayer might provide a novel mechanism for increasing interaction between the liposomal and endosomal membranes, as illustrated in Fig. 6. Inside the endosome, the liposomes might display a net positive charge due to protonation of the anionic lipid CHEMS. Since the endosomal membrane presumably carries a net negative charge, the reversal of the liposomal net charge might lead to liposomal fusion with the endosomal membrane based on electrostatic interactions and release of liposomal content into the cytoplasm. Fluorescence micrographs (Fig. 4) of KB cells exposed to targeted pH-sensitive and non-pH-sensitive liposomes containing calcein provided evidence for the ability of these pH-sensitive liposomes to increase cytosolic delivery of entrapped calcein. The diffuse cytosolic fluorescence occurring in cells exposed to pH-sensitive liposomes, as opposed to the punctate fluorescence seen with non-pH-sensitive liposomes, indicated release of calcein into the cytoplasm.

Since the FR is highly amplified in a wide range of human tumors while possessing a narrow normal tissue distribution, it has been investigated as a target for the selective delivery of therapeutic compounds to tumor cells [15]. Uptake of folate-derivatized liposomes has been previously shown to proceed via

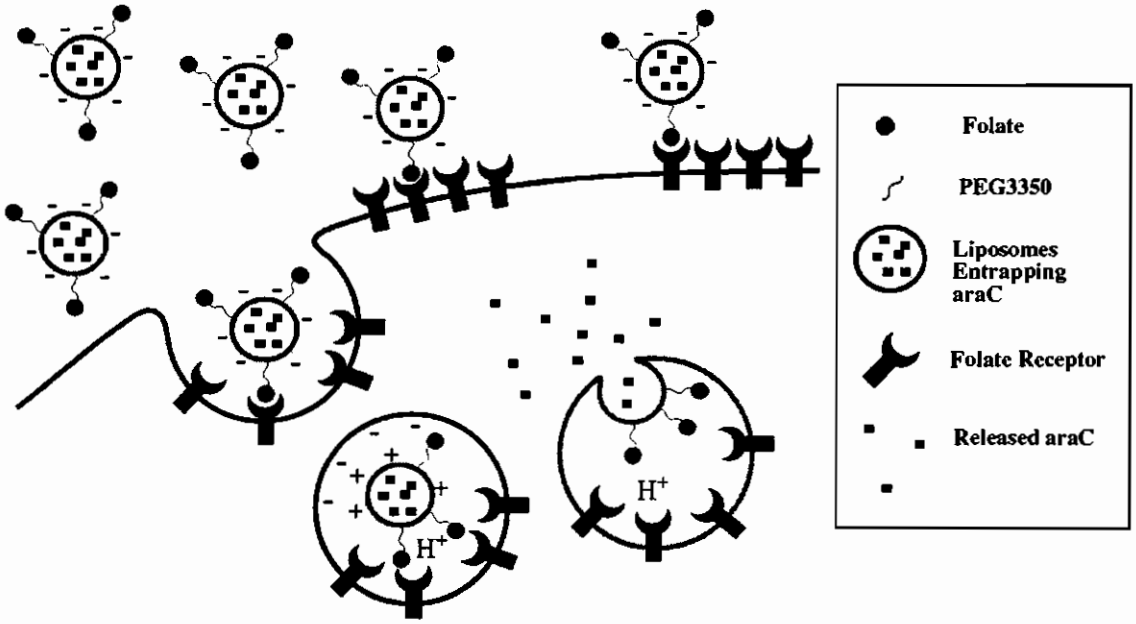

Fig. 6. Possible mechanism of intracellular araC delivery by FR-targeted cationic lipid-based pH-sensitive liposomes. At first, the folate-derivatized liposomes are taken into the cell via binding to the FRs on the plasma membrane and FR-mediated endocytosis. This is followed by acidification of the endosome, which results in protonation of the anionic lipid component and generation of a net positive surface charge on the liposomes. Finally, the electrostatic interactions between the liposomal and endosomal membranes result in bilayer fusion and the cytosolic delivery of the encapsulated araC.

receptor-mediated endocytosis in FR-bearing tumor cells [8,9]. In addition, the endosomal compartment in this delivery pathway has been shown to be highly acidified [11]. Therefore, FR-targeting of pH-sensitive liposomes could provide an ideal mechanism for cell-specific delivery of therapeutic agents that are membrane impermeable.

The cytotoxicity data clearly indicate that optimal activity of liposomal araC required both FR-targeting and pH-sensitive liposome formulation. Interestingly, araC delivered by non-targeted non-pH-sensitive liposomes showed greatly reduced cytotoxicity compared to free araC. A possible explanation is that free araC can access the cell via a nucleoside transporter, whereas liposomal encapsulation without FR-targeting adds an additional barrier to cellular uptake. The relatively high IC_{50} value of free araC might be due to the short incubation time (2 h) used, combined with the fact that araC is an S-phase specific agent. Liposomal araC is expected to have greatly extended systemic circulation time compared to free araC. It might, therefore, have the added advantage of prolonging tumor cell exposure to the drug during in vivo administration, which might lead to improved therapeutic efficacy.

In addition to drug delivery, pH-sensitive liposomes can be used with polycation-condensed DNA to construct LPDII gene transfer vectors [14]. Since fusogenicity rather than drug retention is the key for efficient gene delivery, liposomes composed of DDAB/CHEMS (30:70) were evaluated as the lipidic component for formulating LPDII vectors. Targeted pH-sensitive vectors attained similar levels of transfection to DOPE-based LPDII vectors. In contrast to DOPE-based vectors, which were completely inactivated by serum, the cationic/anionic lipid-based LPDII vectors were highly efficacious in transfecting cells in the presence of serum. These vectors also exhibited FR-dependent gene transfer that could be blocked with free folate.

In summary, FR-targeted pH-sensitive liposomes based on cationic/anionic lipid pair, with the appropriate composition, can facilitate highly efficient intracellular drug and gene delivery. In contrast to

DOPE-based pH-sensitive liposomes, the pH-sensitivity of cationic/anionic liposomes is maintained in the presence of serum. Since retaining activity in the presence of serum is required for use in systemic delivery, these liposomes might potentially be suitable for systemic administration. Further studies are warranted to evaluate the potential use of these formulations for in vivo drug and gene delivery.

Acknowledgements

This work was supported in part by a grant (RPG-99-097-01-MGO) from the American Cancer Society and grant R01 CA79758-01A1 from the National Institute of Health.

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EXHIBIT 20

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May 11, 2008

Manfred Schwab, Dr.rer.nat., Professor of Genetics
Editor-in-Chief, Cancer Letters
German Cancer Research Center (DKFZ)
Im Neuenheimer Feld 280
D-69120 Heidelberg, Germany

Dear Dr. Schwab:

During the course of a university investigation, I became aware of e-mail correspondence dated March 5, 2008 between you and Dr. H.B. Kostenbauder regarding the subject "publishing behavior" and allegations made against me. The exact nature of the allegations sent to you were not included in the materials, although I can assume they are similar to ones found in anonymous letters sent to the Ohio State University in late November 2007.

I am dismayed and disappointed that I was not asked to respond to the allegations sent to you before any decisions were made. The allegations are misleading and provided only selected information. Furthermore, the allegations claim repetition of figures without citation and repeated presentation of same figures and data as apparent new material. These allegations are false.

These false allegations are directed at reviews published from my laboratory. Three of the reviews were published in the *Journal of Steroid Biochemistry and Molecular Biology* in 2001, 2003 and 2005. These manuscripts are review articles and summarize presentations at the International Aromatase Conferences in 2000, 2002 and 2004. The issues of the journal are Special Issues of the proceedings of the three international conferences, and the footnote on the first page of each publication indicates that this review was presented at the particular conference. Presenters at the international conferences were asked to discuss recent research, both published and unpublished work. The subsequent proceedings' manuscripts (reviews) are derived from the presentations. My reviews contain summaries of our recent research publications related to the session of the conference. My presentations and subsequent review articles also contain figures of preliminary research findings that were not yet published. **In the text of the reviews, I cite our primary research papers that were published or in press at the time of the meetings and subsequent review publications.** The fourth review cited is an invited review that appeared in the journal *Anti-Cancer Agents in Medicinal Chemistry* in 2006. This review appeared in a special issue on the topic of cyclooxygenase-2 inhibitors in cancer, and the issue contains five review articles. **In the text of all the reviews, I cite our published primary research papers.** Moreover, a review article by definition discusses results from previous studies. A review article describes the same results and similar conclusions to the previously published manuscripts.

Detailed discussions related to the allegations are presented on the pages following this letter. These detailed discussions were provided for the university investigations and likely address the allegations sent to you and *Cancer Letters*, since I do not know the specifics sent to you. Also, I welcome the opportunity to provide you with any additional information you may need, including PDF files of all the articles with highlighted citations and copies of my presentations at the International Aromatase Conferences.

Furthermore, I ask you to consider the motives of Dr. H.B. Kostenbauder in this matter. Although Dr. Kostenbauder is not a faculty member at Ohio State University, he is the spouse of Dr. Sheryl Szeinbacb who is a faculty member in the College of Pharmacy at Ohio State. Another faculty member submitted allegations

via the OSU "whistleblower report form" in May 2007 that Dr. Szeinbach published one manuscript in 2005 and another in 2007 in different journals with no citation to the 2005 article, implying the appearance that the 2007 manuscript is new material. As required by University guidelines, I as the college administrator was a member of the initial committee of four individuals who reviewed the report form and subsequently referred the matter to a confidential faculty committee of inquiry for a full investigation. During the university investigations, Dr. Szeinbach published a correction and the editor of the journal containing the 2007 article published an editorial on the matter, both appearing in August 2007. A simple question – is there any connection of the Szeinbach investigation to any misleading or false allegations that have been made against myself and others involved in the University process?

Finally, it is customary and fair that the accused be asked to respond to allegations before any decisions are made. Therefore, I am requesting, as professional courtesy, that this letter and my response be fully considered before any final decisions are made.

Sincerely,

Robert W. Brueggemeier

Robert W. Brueggemeier, Ph.D.
Professor and Dean, College of Pharmacy